THE UTILIZATION OF LACTOSE BY THE MATURE FOWL[1]

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Guha ('31) reported that rats rapidly declined in weight and died when galactose was used as the carbohydrate in a fat-free diet. Bilateral cataract was observed in rats fed 70% lactose by Mitchell and Dodge ('35). Schantz et al. ('38) fed rats on mineralized skim milk and found sugar in the urine, which was identified as galactose; similar animals fed mineralized whole milk made efficient utilization of the milk sugar. These workers noted that 3 to 4% fat in the mineralized skim milk diet prevented the loss of sugar in the urine. In a further study Schantz and Krewson ('39) reported that even chain fatty acids with over 12 carbon atoms fed at a rate of 3 to 4% were effective in preventing the loss of galactose in the urine of rats fed mineralized skim milk, while such fatty acids of less than 12 atoms were not effective in preventing this loss.

It was reported by Couch et al. ('48) that the feeding of 15% lactose in a purified diet to laying hens appeared to have a deleterious effect on egg production after about three weeks. Twenty-five per cent dried whey (equivalent to 15% lactose) had a similar effect. It was noted further that neither

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lactose nor dried whey promoted the intestinal synthesis of biotin.

The present investigation was initiated to study the utilization of lactose by the laying hen. It was deemed advisable to use 63% lactose since it was thought that the use of this sugar as the only carbohydrate in a purified diet would give more accurate data on its utilization by the laying hen.

MATERIALS AND METHOD

Single-comb White Leghorn pullets reared on the Experiment Station range were placed in individual laying cages with raised screen bottoms. All birds were artificially inseminated weekly with mixed semen from New Hampshire cockerels. All eggs laid were marked with the hen's number and date. Settings were made weekly. The eggs were candled on the 7th and 17th days of incubation, at which times the infertile eggs and dead embryos were removed. All eggs which were removed on candling, and also those which failed to hatch, were broken and the age of the embryo at the time of death was estimated.

The composition of the experimental diets is given in table 1. Five experiments were conducted. Four birds were included in all groups. In experiment 1 diets B36 and B37 were fed for a 4-week period. Experiment 2 included diets B36A and B41–44A. At the end of the first two weeks diets B43 and B44 were discontinued and two hens from each of these groups were fed diet B44A for the remainder of the experiment. Diet B34 served as a control diet for this 7-week experiment. In experiment 3 diets B36A, B44A and B50 were fed for a 5-week period. B34 again served as the positive control. The birds were continued on diet B36A and 44A for the metabolism studies outlined below after experiment 3 was terminated.

Two metabolism experiments were carried out. In the first case 4 birds were fed the practical all-mash diet (Diet B1 of Robblee et al., '48) ad libitum and in addition two of these birds were given by capsule 25 gm of a 1:1 mixture of galac-

tose and glucose, while the remaining two were given 25 gm of lactose. Blood was obtained by puncturing the wing vein. Blood galactose determinations were made on individual hens each hour for 4 hours after the administration of the sugar. In the second metabolism experiment one group of 4 hens

TABLE 1

Composition of experimental diets (parts by weight)

COMPONENTS	DIET									
	B34	B36	B36A	B37	B41	B42	B43	B44	B44A	B50
	Dietary components kept constant									
Casein purified	18	18	18	18	18	18	18	18	18	18
Gelatin	5	5	5	5	5	5	5	5	5	5
Salts IV	5	5	5	5	5	5	5	5	5	5
Liver fraction L	4	4	4	4	4	4	4	4	4	4
Fish oil (3000 A, 400 D)	2	2	2	2	2	2	2	2	2	2
Choline	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Crystalline vitamins [1]										
Oyster shells — ad libitum										
	Dietary components that were varied									
Sucrose	63									
Whole milk powder										100 [2]
Lactose		63	63	63	60	58	63	53	38	
Soybean oil	3	3	3		3	3	3	13	28	
Butter oil				3						
Fish solubles					3					
Whole liver powder						5				
Desiccated thyroid							0.125			
Biotin (mg/kg)	0.2		0.2		0.2	0.2	0.2	0.2	0.2	

[1] Crystalline vitamins, in milligrams per kilogram of ration, added to all diets as follows: Thiamine-HCl 4; riboflavin 6; Ca-pantothenate 15; niacin 100; menadione 0.5; pyridoxine-HCl 4; alpha-tocopherol 4.

[2] Fortified haliver oil (60,000 A, 10,000 D) two drops per hen daily. Also, in milligrams per kilogram of ration: Iron 20, copper 2, manganese 50, and iodine 1.

was fed diet B36A and a second group of 4 was fed diet B44A ad libitum. Two birds from each group were given individual doses of 25 gm of a 1:1 mixture of galactose and glucose, while the other two birds were each given 25 gm of lactose by capsule. Individual blood galactose determinations were made at the end of the first, second, third, 6th and 8th hours.

Blood glucose or total blood sugar was determined according to the method of Folin and Malmros ('29) as modified by Horvath and Knehr ('41). In each instance a separate sample (0.1 ml) of blood was collected for the galactose determination, which was carried out as outlined below. One-tenth milliliter of whole blood was pipetted into 10 ml of sodium tungstate-sulfuric acid solution (Folin and Malmros, '29). After centrifuging, 8 ml of supernatant liquid was pipetted into another centrifuge tube and 0.2 ml of fresh live yeast (prepared by washing 6 to 8 times with water — 1 gm washed yeast made up to 2 ml) added. After incubation at 37°C. for 30 min., an aliquot (4 ml) was taken and was carried through the procedure outlined above for determining total blood sugar. It was found that the yeast failed to remove completely the reducing substances of the blood from the blood of hens fed sucrose (diet B34). Thus it was necessary to use blood from hens fed sucrose as a blank in each series. In this case the milligram per cent of reducing substances in the blood of hens fed diet B34 was calculated in each determination after fermentation with yeast; this value was subtracted from the milligram per cent galactose observed after fermentation of blood from hens fed lactose, or the blood of those fed the 1:1 mixture of galactose and glucose. The figure thus obtained was termed "milligram per cent galactose."

EXPERIMENTAL RESULTS

Experiment 1 was designed to test the effect of butterfat and soybean oil on the intestinal synthesis of biotin with lactose as the carbohydrate. As is shown in table 2, neither diet B36 nor B37 supported egg production. Thus this experiment yielded no data on the relation of fat to intestinal synthesis,

but its results are included here to show the effect on egg production of diets containing lactose as the sole carbohydrate.

The egg production and hatchability data obtained in experiments 2 and 3 are presented in table 2. It may be seen that the rate of production of the birds fed diet B36A fell rapidly. These birds ceased to lay after the second week of the experiment. The addition of fish solubles (B41) or des-

TABLE 2

Effect of diets on egg production and hatchability

EXPERIMENT NO.	DIET NO.	EGG PRODUCTION								HATCHABILITY
		Pre-expti.	1 week	2 week	3 week	4 week	5 week	6 week	7 week	
		%	%	%	%	%	%	%	%	%
1	B36	—	46	0	0	0				80
	B37	—	46	0	0	0				67
2	B34	79	79	68	70	79	79	64	71	90
	B36A	75	43	6	0	0	0	0	4	100
	B41	75	43	21	4	4	0	0	4	89
	B42	68	35	10	10	18	35	21	14	75
	B43	75	39	0 [1]	—	—	—	—	—	100
	B44	61	21	0 [1]	—	—	—	—	—	76
	B44A			[2]	0	26	57	53	43	90
3	B34	68	71	58	64	64	57			70
	B36A	85	47	10	3	0	0			100
	B44A	85	48	57	64	64	57			84
	B50	75	39	18	39	53	43			91

[1] Discontinued.

[2] Two hens from diet B43 and two from B44 given diet B44A.

iccated thyroid (B43), or an increase in the level of soybean oil to 13% (B44) failed to result in improved egg production. The addition of 5% whole liver powder to diet B36 (B42) resulted in some improvement in production, although it is obvious that production was significantly below that obtained with the control diet, B34.

It has been mentioned earlier that the groups fed diets B43 and B44 were discontinued at the end of the second week; the per cent egg production of these groups was zero at this time.

Two hens were selected from each of those groups at the end of the second week and were fed diet B44A (lactose with 30% fat) from the beginning of the third through the 7th week of the test. The egg production of hens fed diet B44A increased, and approached that of those fed B34. From these results it is apparent that 30% fat with lactose (diet B44A) tended to correct the deleterious effect of lactose alone on egg production when fed to the laying hen.

Further evidence that a high level of fat counteracts the deleterious effect of lactose on laying hens is shown in the results of experiment 3. Although production was somewhat erratic, due to the small number of birds in each group, it is obvious that diet B44A supported production at a rate equal to that of the control diet, B34. Egg production of the birds fed dried whole milk (B50) decreased during the first two weeks of the experiment, then increased to a level somewhat below that obtained with the control ration. The whole milk powder was quite hygroscopic and tended to gum up in the beak as it was eaten. One hen of the group refused to eat an appreciable quantity of the diet and laid no eggs after the first week of the experiment. The weight of this bird decreased approximately two-thirds during the experiment, whereas the other hens fed the whole milk powder gained from 200 to 400 gm each during the test. The body weights of hens fed diet B36A fluctuated within about 100 gm of the starting weight in each instance.

The hatchability data for all groups of birds, given in table 2, show that there is no significant difference in this respect in any of the rations fed in these experiments.

The results of the metabolism studies are given in table 3. These data indicate that the presence of 30% fat in a lactose diet aids in the utilization of this carbohydrate. This is apparent when the figure for milligram per cent of galactose in the blood of hens fed the practical diet is compared with that of the hens fed diet 44A, where both groups had received a 25 gm dose of the 1:1 mixture of glucose and galactose. The practical diet contained 5 to 6% fat and when

hens were fed this diet and given 25 gm of the sugar mixture, the blood galactose level was higher throughout the 4-hour period than that of hens fed a lactose diet containing 30% fat. Similar and more convincing results were obtained when the glucose-galactose mixture was fed to one group of hens receiving a ration containing 5% fat (B36A) and to another receiving a diet containing 30% fat (B44A).

TABLE 3

Effect of fat on blood galactose level after administration of 25 gm of lactose or 25 gm of a 1:1 mixture of glucose plus galactose

HEN DIET	SUGARS FED	MG % GALACTOSE AT HOURS AFTER FEEDING							
		1	2	3	4	5	6	7	8
Practical	Lactose	0	20	7	20				
	Glucose + galactose	80	150	148	212				
B44A (30% fat)	Lactose	36	18	18	13				
	Glucose + galactose	62	91	140	165				
B36A (5% fat)	Lactose	0	18	9				8	8
	Glucose + galactose	76	124	170				193	191
B44A (30% fat)	Lactose	0	30	5				5	5
	Glucose + galactose	42	60	82				64	42

In view of the fact that the feeding of lactose in a purified diet (B36A) stopped the hens from laying in such a short time, it was thought that galactose in the blood of the fowl might in some manner be interfering with the secretion of the hormones of the anterior pituitary, which control egg production. However, when hens were fed diet B36A and injected in one case with a preparation of the luteinizing hormone and in another with a preparation of the anterior pituitary, egg production of birds receiving the injections was about the same as that of those fed diet B36A without injections. It was noted that injection of the hormones caused a decided decrease in weight (500 to 600 gm per bird in two weeks).

DISCUSSION

The egg production data show clearly that the presence of fat in the diet enables the hen to use high levels of lactose, as is indicated by continued egg production.

The effect of fat could be considered as a mere replacement of lactose in the diet. If such is the case, then the addition of sucrose to a lactose diet should have a similar effect, but previous work with a diet containing 15% lactose (Couch et al., '48-diet B33) showed that sucrose in amounts isocaloric to the fat in the present experiments did not prevent this deleterious effect of lactose on egg production.

It was mentioned above that Schantz et al. ('38) observed galactosuria in rats fed skim milk, but when the skim milk was supplemented by 3 to 4% fat the loss of sugar in the urine was prevented. Geyer et al. ('45), also using galactosuria as a criterion, concluded that fat increases the utilization of galactose. Nieft and Deuel ('47) found that the rate of intestinal absorption of galactose in the rat varies inversely with the percentage of fat in the diet. They suggest that the galactosuria may be a "flooding effect" produced by the faster absorption rate of galactose, and that the slowing of this absorption by the fat enables the rat to metabolize the galactose more completely, thus preventing galactosuria.

While our short period blood sugar studies do not indicate an effect of fat on the galactose level of the blood when lactose is fed, the data obtained from the hens fed the mixture of glucose and galactose show that the presence of high fat in the diet results in lower blood galactose levels. This effect might be due, as suggested by Nieft and Deuel, to a slower absorption rate and the prevention of "flooding." It may also be a metabolic effect produced after the galactose reaches the blood. Further work is being carried out in our laboratory to study this possibility.

SUMMARY

Laying hens fed a purified diet with lactose as the carbohydrate ceased laying in one to two weeks after being placed

on the diet. Thirty per cent fat in the diet corrected the deleterious effect of lactose on egg production. High levels of lactose appeared not to affect hatchability.

In metabolism studies high levels of fat in the diet resulted in lower blood galactose levels when galactose was administered orally.

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RELATION OF IODINE TO THE GOITROGENIC PROPERTIES OF SOYBEANS [1]

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FOUR FIGURES

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Various investigators have reported that soybeans contain a goitrogenic agent which increases the iodine requirement of chicks and rats. McCarrison ('33) reported a high incidence of thyroid enlargement in rats fed a diet containing raw soybeans. His results indicated that raw soybeans produce goiter even when iodine is fed in large amounts. Sharpless et al. ('39) reported that soybean flour contains a positive goitrogenic property that is partially removed or destroyed by fat solvents (ether or acetone) or by steam. Wilgus et al. ('41) also found that the soybean agent was partially inactivated by heat, but they were unable to extract the factor with solvents similar to those employed by Sharpless. Both groups of investigators showed that the addition of small amounts of iodine to the goitrogenic diets will largely prevent the thyroid enlargement. They also showed that the goitrogenic property of soybeans is not due solely to their low iodine content. Wilgus et al. found the iodine content of a ration containing 15% casein to be approximately the same as that in

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the goitrogenic soybean rations, yet the casein ration caused only minor thyroid enlargement in comparison to the soybean rations. Sharpless et al. adjusted the iodine content of a soybean diet by supplementation to a level comparable to that of the stock ration and then measured the thyroid size and iodine content of glands from animals in both experimental and control groups. The thyroid glands of the stock animals were normal but the glands from the soybean rats were enlarged and low in iodine.

The rachitic ration of Steenbock and Black ('25), which was modified by Levine et al. ('33), has been reported by several investigators (Krauss and Monroe, '30; Thompson, '32; Hall, '46) to produce goiter in rats. The composition of this ration, as modified, follows: yellow corn, 76 parts; wheat gluten, 20 parts; $CaCO_3$ three parts; NaCl, one part; and irradiated yeast, 0.2 part. Levine et al., Thompson, and Hall have shown that the goitrogenic properties of this ration are prevented by small amounts of iodine. In cases where this diet fails to produce goiter, either the ration contains significant amounts of iodine or the rats employed in the experiment have a high initial store of reserve iodine. Levine et al. also showed that 1 to 2 μg of iodine are required per day to maintain normal thyroid structure in the growing rat. The requirement was determined by comparing the weight, iodine content and dry matter content of thyroid glands from rats supplemented with various levels of iodine (KI). Most investigators accept the iodine requirement of the growing rat as determined by these workers as being the normal value. The question of whether this requirement is altered by diverse types of diets has not been adequately investigated. The fact that animal proteins such as casein (Remington, '37) generally contain significant amounts of iodine has prevented their general use in iodine requirement studies.

EXPERIMENTAL

The effect of iodine supplements upon the size, iodine content and histological structure of the thyroid gland was

studied with rats fed various types of soybean-containing rations. In the first experiment two groups of rats (6 per group) were fed a soybean ration to test the effect of soybeans in causing increased thyroid enlargement. One of the groups received no added iodine and the other received an iodine supplement of 2.0 μg of iodine per rat per day. The soybean ration consisted of 10 parts of raw soybeans added to 90 parts of the basal ration.

Rations

The basal ration selected was similar to the previously described diet employed by Levine et al. ('33). The percentage composition of the ration follows: wheat gluten, 30; ground yellow corn, 68; NaCl, 1; and $CaCO_3$, 1. Reagent grade NaCl and $CaCO_3$ were used in the rations. The water-soluble vitamins were dry mixed with the wheat gluten before the ration was prepared. The vitamin levels added per kilogram of ration follow: 6.0 mg thiamine hydrochloride, 9.0 mg riboflavin, 9.0 mg pyridoxine hydrochloride, 60.0 mg calcium pantothenate, 20.0 mg nicotinic acid, 300.0 mg *p*-aminobenzoic acid, 1.0 gm inositol, 2.0 gm choline chloride, 0.1 mg biotin and 0.3 mg folic acid. The fat-soluble vitamins were dissolved in corn oil [2] and fed by dropper once a week. The amount of each of the fat-soluble vitamins fed per rat per week was: 3.0 mg α-tocopherol, 10.0 μg calciferol, 0.15 mg 2-methyl-1, 4-naphthoquinone and 0.16 mg β-carotene.

Great care was used in the preparation of the experimental rations. The corn and soybeans were ground in a burr mill which had been thoroughly washed previous to use. The rations were mixed in a porcelain jar by stirring with a glass rod.

The conditions employed in the second experiment (table 1, expt. 2) were similar to those of the first except that a salt mixture was substituted for the $CaCO_3$ and NaCl in the basal ration. A supplementary group (6 rats) which received the

[2] Mazola.

TABLE 1

Iodine requirement of rats fed raw and heated soybeans[1]

EXPERIMENT NO.	SUPPLEMENT		TOTAL IODINE INTAKE PER RAT PER DAY	BODY WEIGHTS: INITIAL AND FINAL	THYROID WEIGHTS (FRESH) PER 100 GM BODY WEIGHT		IODINE CONTENT OF THYROID		
							Total		On dry weight basis
	Soybean	Iodine			Average	Range	Average	Range	
	%	μg	μg	gm	mg	mg	μg	μg	%
1	None	None	0.26	42–151	18.5	14.8–20.2	0.6	0.5–0.78	0.009
	None	0.5	0.76	41–148	10.8	9.0–12.5	2.7	1.4–4.4	0.06
	None	1.0	1.26	42–146	11.3	9.9–12.2	3.4	2.6–4.1	0.08
	None	2.0	2.26	43–154	10.2	8.9–11.4	9.0	5.5–13.5	0.18
	10 raw	None	0.30	42–144	17.1	14.1–19.4	0.7	0.6–0.8	0.01
	10 raw	2.0	2.30	43–146	11.6	10.3–13.2	9.3	4.8–13.2	0.18
2	None	None	0.25	43–175	24.1	16.7–36.5	0.18	0.13–0.22	0.0017
	30 raw	None	0.33	44–155	27.8	23.0–39.6	0.21	0.13–0.28	0.0026
	30 raw	0.5	0.83	44–150	15.0	12.0–17.5	3.0	2.0–3.6	0.04
	30 raw	1.0	1.33	44–158	12.8	9.8–17.6	4.6	3.7–6.3	0.07
	30 raw	2.0	2.33	43–154	12.8	10.4–15.3	8.2	4.2–11.2	0.13
	30 heated	None	0.36	43–177	25.3	15.1–44.0	0.23	0.18–0.26	0.0025
	30 heated	0.5	0.86	42–166	14.0	10.3–17.1	2.9	1.5–4.4	0.04
	30 heated	1.0	1.36	42–176	12.7	10.9–13.9	5.3	3.6–7.0	0.08
	30 heated	2.0	2.36	43–165	10.0	8.6–11.3	7.6	5.9–11.0	0.14

[1] Figures represent the average of 6 rats.

salt mixture in place of $CaCO_3$ and NaCl had previously been included in experiment 1. The purpose of this group was to determine whether the addition of a more complete mineral supplement would alter the goitrogenic property of the ration. Since the thyroids of the rats receiving the salt mixture were enlarged to the same extent as those receiving $CaCO_3$ and NaCl, it was decided to add the salt mixture to the basal ration in experiment 2.

The percentage composition of the basal ration employed in experiment 2 follows: ground yellow corn, 66; wheat gluten, 30; and salts, 4. The percentage composition of the salt mixture prepared from reagent grade chemicals was: $Ca_3(PO_4)_2$, 48; NaCl, 30; KCl, 10; $MgSO_4 \cdot 7H_2O$, 8.0; $FeCl_2 \cdot 4H_2O$, 3.2; $MnSO_4 \cdot H_2O$, 0.6; $ZnSO_4 \cdot 7H_2O$, 0.1; and $CuSO_4 \cdot 5H_2O$, 0.1. The $Ca_3(PO_4)_2$ was further purified by a procedure described in an earlier paper (Halverson et al., '45). The water-soluble and fat-soluble vitamins were fed at identical levels and in the same manner as in experiment 1.

The level of soybeans added to the basal ration was increased to 30% since no significant goitrogenic effect had been observed with 10% in experiment 1. The heated soybeans were prepared by autoclaving the raw ground beans in thin layers in open glass dishes for 10 min. at 15 lb. pressure. The soybeans employed in both experiments were the Kingwa variety.

The low iodine diets were supplemented with potassium iodide (aqueous solution) by dropper. Levels of 0.5, 1.0 and 2.0 μg of iodine per rat per day were fed the various groups. Since the daily iodine requirement of the growing rat has been reported to be 1 to 2 μg, these levels of supplementation were thought to be the most desirable in order to study any goitrogenic effects.

Experimental animals

Female albino weanling rats (39 to 49 gm) of the Sprague-Dawley strain were employed and 6 animals per group were

used throughout the studies. The animals were housed in individual wire cages. The double distilled water and the experimental rations were fed in porcelain dishes. The growth rates of the rats were followed by weighing the individual animals at weekly intervals. Food consumption was measured frequently.

The animals were kept on the experimental diets 7 weeks, at which time they were killed by ether anesthesia. The thyroids, together with the parathyroid glands, were removed with the aid of a binocular dissecting microscope and weighed immediately on a torsion balance. This balance weighed to 0.1 mg and little, if any, blood or extraneous tissue was left attached to the thyroid. The thyroids used for histological study were prepared in the usual manner using Bouin's fixative and staining with hematoxylin and eosin. In experiment 2 the thyroids from half of the animals of each group were used for histological examination and the remaining thyroids (three from each group) were dried, weighed and saved for iodine analysis. All of the thyroids of animals used in experiment 1 were dried, weighed and saved for iodine analysis.

Iodine determinations

The Chaney method was selected for the iodine determinations because of its simplicity and dependability. Numerous investigators (Leipert, '33; Trevorrow and Fashena, '35; and Matthews et al., '38) have shown that small amounts of iodine can be quantitatively recovered by distillation from a digestion mixture of chromic and sulfuric acids after the addition of a suitable reducing agent. Because of the extreme sensitivity and specificity of the catalytic action of the iodide ion on the reduction of the ceric ion by arsenious acid, Chaney ('40) and Taurog and Chaikoff ('46) have adapted this reaction to the measurement of the small amounts of iodine present in the distillate mentioned above. The procedure followed for measuring the iodine in the thyroid glands was identical with that described by Taurog and Chaikoff, who

used the Chaney method for the determination of plasma iodine. Recoveries were run with inorganic iodine (KI) to determine the percentage recovery with amounts of iodine at levels comparable to those in the thyroid glands. The recoveries from 0 to 10 μg iodine ranged between 65–100%. Suitable correction factors were employed in the gland analysis to correct for the losses determined by the recoveries.

The iodine analyses of the low-iodine rations required an initial combustion. The method of Karns ('32), as modified by von Kolnitz and Remington ('33), was employed. After combustion of the sample and collection of the absorbing liquid and rinsings, the analyses were performed by the same procedure as that used for the iodine in the glands. The recoveries, which were run by burning samples of ration containing added potassium iodide (0 to 4 μg), averaged 102.8% (81 to 121).

The method was found to be readily applicable to the estimation of thyroid iodine. However, it was more difficult to reproduce results with the rations (combusted samples). The minerals present in the absorbing liquids often caused a precipitation to occur in the digestion flasks. Sufficient analyses were run on the rations and on the rations plus added iodine to enable the iodine values to be averaged. The principal difficulty encountered in determining the iodine content of the various rations was that the values obtained for those containing the salt mixture were approximately three times as high as those obtained on rations containing $CaCO_3$ and NaCl. The thyroid enlargement on the two rations on the same experiment was very similar (18.5 and 20.2 mg per 100 gm of body weight). On the basis of thyroid enlargement it was concluded that the two rations were very nearly the same in iodine content, even though the values obtained by chemical analysis were different. The iodine values obtained with the ration containing the salt mixture could not be correct, for no appreciable thyroid enlargement would have been obtained at such a high level of iodine intake.

The individual constituents of the rations employed in experiments 1 and 2 were then analyzed for iodine. The iodine content of the basal ration of experiment 1 was the same when the $CaCO_3$ and NaCl were removed as when they were present. In the case of the basal ration of experiment 2, the iodine content was the same as that of the basal ration of experiment 1 when the salt mixture was removed. The salt mixture was then analyzed separately and found to give an iodine value of the same magnitude as that calculated from the difference between the basal ration with and without the added salt mixture. It would appear from these observations that the high iodine results with the rations containing the salt mixture were due to contamination by some interfering ion present in the minerals. Several ions such as osmium, mercury and silver have been shown by Sandell and Kolthoff ('37) to interfere with the catalytic effect of iodides on the reduction of the ceric ion by arsenious acid. It is possible that traces of an interfering ion may be present in commercial mineral preparations. It is also possible that interfering substances may exist in natural feedstuffs in amounts sufficient to invalidate the results obtained when they are used; but since the iodine values obtained on the natural feedstuffs were very low and within the range expected, they were assumed to constitute an accurate measure of iodine content. It is felt that the iodine values obtained for the rations used in experiment 2 were approximately correct, even though salts were not added to the rations analyzed.

RESULTS

The groups fed rations supplemented with potassium iodide showed no significant differences in food consumption or rate of growth from those which received no iodine supplements. The rats of all groups appeared healthy and normal throughout the experiments.

In experiment 1 (table 1) the effects of iodine supplements upon thyroid weight (fresh weight) and iodine content (dry basis) are shown. Supplementing the basal ration with 0.5 μg

of iodine per rat per day effectively prevented thyroid enlargement; however, the per cent of iodine in the dried glands was less than 0.1%. Marine ('22) found that when the iodine concentration in the thyroid falls below 0.1% (dry basis), hyperplastic changes are regularly present. Supplementing the basal diet with 1.0 and 2.0 μg of iodine per rat per day increased the average iodine content to 0.08 and 0.18%, respectively. The addition of 10% of raw soybeans to the basal ration did not significantly change the thyroid enlargement from that observed on the basal ration alone. When the soybean ration was supplemented with 2.0 μg of iodine per rat per day, the thyroids were slightly larger than those of animals on the basal ration receiving the 2.0 μg supplement (11.6 and 10.2 mg, respectively, per 100 gm of body weight). The average iodine contents of the glands from both groups receiving the 2.0 μg supplement were the same (0.18%).

In experiment 2 (table 1) the thyroids from rats receiving 30% of raw soybeans were slightly larger than those from rats on the basal ration (27.8 and 24.1 mg). Thyroids from rats receiving the heated soybean ration (30% heated soybeans and 70% basal ration) were approximately the same size as those from animals fed the basal ration (25.3 and 24.1 mg, respectively). When the rations of rats receiving raw and cooked soybeans were supplemented with 0.5 μg of iodine per rat per day, their thyroids were still enlarged (15.0 and 14.0 mg, respectively) and the average iodine content of the thyroids was below normal (0.04%). An iodine supplement of 1.0 μg reduced the thyroid size to 12.8 and 12.7 mg, respectively, for the raw and cooked soybean rations and increased the average iodine content of the glands to 0.07 and 0.08%. The 2.0 μg iodine supplement did not further reduce the thyroid size (12.8 mg) for the group receiving raw soybeans, but the thyroids from the cooked soybean group were reduced in size (10.0 mg). The average iodine contents of the 2.0 μg raw and cooked soybean groups were 0.13 and 0.14%, respectively.

Histological examination of thyroids from animals on the basal diet without added soybeans revealed epithelial hyper-

trophy and mild hyperplasia (fig. 1). There was little colloid present in these thyroids except in the periphery of the lobe. Foci of beginning necrosis were common in the thyroids from the control animals. Thyroids from the groups receiving 30% of raw or heated soybeans with no iodine supplement showed a more severe epithelial hyperplasia and more numerous foci of necrosis (fig. 2). However, no difference in pathology of the thyroids could be detected between animals receiving raw or heated soybeans. There was a reduction in the severity of the epithelial hypertrophy and hyperplasia when the rations of animals receiving raw and heated soybeans were supplemented with 0.5 μg of iodine per rat per day (fig. 3). The acini were generally small but they did contain a pale staining colloid. Foci of necrosis were still frequent in the thyroids of these groups and many acini were found to contain, in addition to the colloid, several pyknotic nuclei and leukocytes within their lumina. The administration of 1.0 or 2.0 μg of iodine was effective in reducing the thyroid pathology in the animals receiving the soybean diets (fig. 4). Histologically, most of these thyroid acini were normal in size and were filled with a dense colloid. However, significant numbers of leukocytes and pyknotic nuclei were still present within the lumina of these acini, and foci of necrosis were still common. No differences in the severity of the thyroid pathology were observed between the groups receiving raw and heated soybeans at the different levels of iodine supplementation.

DISCUSSION

The results of our experiments indicate that small amounts of iodine can effectively prevent thyroid enlargement in rats on soybean-containing rations. The amount of iodine necessary to prevent gross enlargement on the soybean rations was within the range of the accepted requirement of 1 to 2 μg of iodine per rat per day. The thyroid weight, iodine content and histological data show that the addition of raw or heated soybeans to the basal ration used increased the iodine re-

quirement of rats to some extent. The iodine analysis values indicate that the addition of either raw or cooked soybeans to the diet did not prevent or significantly alter the uptake of iodine by the thyroid gland. The average iodine content was greater than the critical level of 0.1% (dry basis) when the basal and the soybean-containing rations were supplemented with 2.0 μg of iodine per rat per day. Small differences in thyroid weight and iodine content also indicate that a more severe goiter was obtained with 30 than with 10% raw soybeans and with raw than with cooked beans. Since no definite histological differences between the groups fed the raw and the heated soybeans were evident, the significance of the slight differences in iodine content and thyroid size between the raw and the heated soybean rations should be appraised with caution.

When 30% of raw or heated soybeans were added to the basal ration, a more severe epithelial hyperplasia and necrosis of the thyroid gland resulted than when rats were fed the basal ration only. The addition of small amounts of iodine (0.5 to 2.0 μg) to the soybean-containing rations resulted in a definite decrease in the severity of the glandular pathology. The colloid accumulation which occurred with iodine supplementation was accompanied by greater iodine content as measured by chemical analysis. Even when the rats receiving the soybean-containing rations were supplemented with 1.0 and 2.0 μg of iodine per day, the gland structure was not completely normal, for areas of necrosis were present. It is not known whether supplementation with amounts of iodine larger than 2.0 μg would completely prevent this necrosis. Since no significant gland enlargement occurred at levels of 1 to 2 μg of iodine per day, it is concluded that the amount of iodine supplied was adequate to enable the thyroid gland to make a sufficient amount of the iodine-containing hormone (thyroglobulin) to supply the normal physiological requirements of the growing rat.

The results obtained are in essential agreement with the data of Sharpless et al. ('39), who found that autoclaved soy-

beans produced less thyroid enlargement than the raw beans. When they fed rats a raw soybean ration containing various levels of added iodine, they also found that the level of iodine necessary to prevent thyroid enlargement was greater than the normal requirement. They did not show whether a definite decrease in iodine requirement resulted from heating the soybeans. Wilgus et al. ('41) reported that unheated soybeans produce much larger goiters in chicks than heated beans. They also noted that small amounts of iodine are effective in lessening the severity of goiter on the soybean-containing rations, but they did not report sufficient iodine supplementation experiments to determine the extent to which the iodine requirement on such rations is increased above the normal. These workers obtained with chicks greater differences in thyroid weights between raw and heated soybeans than Sharpless and associates did with rats. Wilgus et al. examined histologically thyroids from chicks and rats on the same soybean-containing ration and concluded that the chick thyroid is more sensitive to goitrogenic rations than is that of the rat.

The present authors agree with Wilgus et al. that the slight goitrogenic property of raw and heated soybeans is not of practical importance in animal nutrition, since small amounts of iodine can effectively prevent the resulting thyroid enlargement. A ration containing 1% of iodized salt (0.01% potassium iodide) supplies a rat consuming 10 gm of ration per day with 7.6 μg of iodine. Such an amount is adequate to prevent goiter under all ordinary conditions.

SUMMARY

1. The thyroid enlargement obtained with rations containing 10 and 30% of raw or heated soybeans was effectively prevented with 1 to 2 μg of iodine per rat per day. The iodine requirement of rats was slightly greater on soybean-containing rations than on the basal ration employed.

2. The reduced thyroid enlargement resulting from heat treatment indicates that the goitrogenic property of soybeans is partially heat labile.

3. Since small amounts of iodine effectively correct the mild goitrogenic property of soybeans, no harmful effects should result from the use of soybeans in practical rations.

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PLATE 1

EXPLANATION OF FIGURES

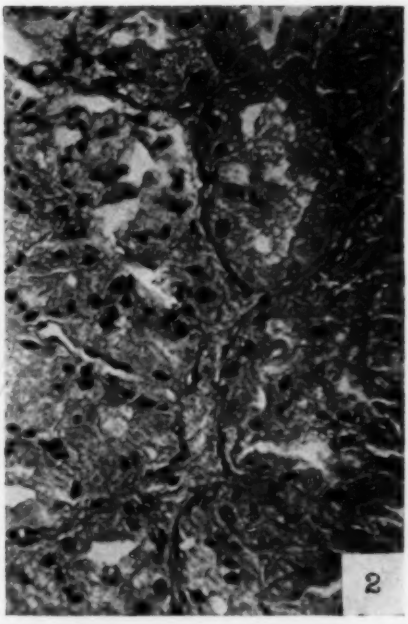
1 A section of the thyroid gland of a rat which received the basal ration only. Epithelial hypertrophy, hyperplasia and necrosis. Hematoxylin and eosin ×350.

2 A section of the thyroid gland of a rat which received the basal ration with 30% raw soybeans. Epithelial hypertrophy and more severe necrosis. No difference in the pathology of the thyroids could be found between animals receiving the raw or cooked soybeans. Hematoxylin and eosin ×350.

3 A section of the thyroid gland of a rat which received the basal ration containing 30% cooked soybeans plus 0.5 μg of iodine per day. Small acini filled with colloid and mild necrosis. No difference in the pathology of the thyroids could be found between animals receiving the row or cooked soybeans. Hematoxylin and eosin ×350.

4 A section of the thyroid gland of a rat which received the basal ration with 30% cooked soybeans plus 1 μg of iodine per day. A few pyknotic nuclei and lymphocytes within the colloid-filled lumina. Supplementation with 2 μg of iodine per rat per day gave the same histological picture of the thyroid as did supplementation with 1 μg of iodine. Hematoxylin and eosin ×350.

1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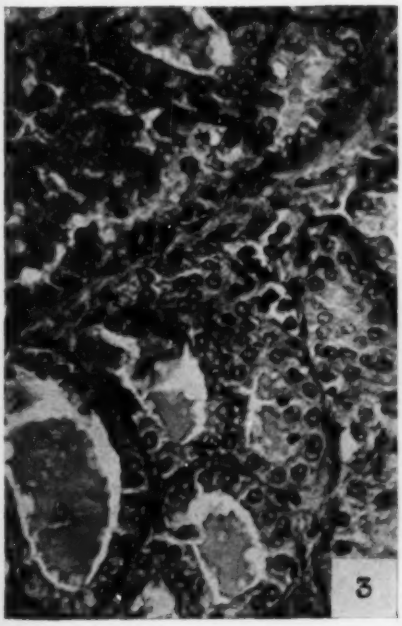
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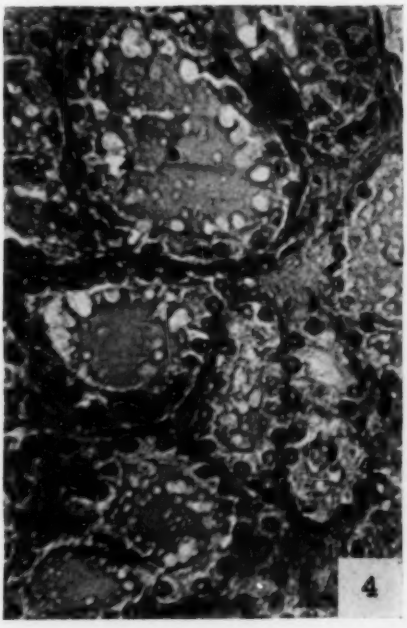
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VITAMIN B_6 DEFICIENCY IN THE SYRIAN HAMSTER [1]

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TEN FIGURES

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While vitamin B_6 deficiency has been investigated extensively in the rat and other animal species (i.e., dog, chick, swine, monkey and mouse), there have been few and inconclusive observations made on this deficiency in the Syrian hamster. Routh and Houchin ('42) reported briefly an "acrodynia-like" dermatitis around the mouth, loss of weight and death within 24 days in hamsters fed a pyridoxine-free diet. Cooperman, Waisman and Elvehjem ('43) and Hamilton and Hogan ('44) included pyridoxine in their experimental diets for hamsters, but made no analysis of B_6 deficiency. The experiments embodied in this paper are concerned with the production of vitamin B_6 deficiency in the Syrian hamster.

EXPERIMENTAL

Methods

With the exception of the preliminary experiment, the work was done on male Syrian hamsters of weanling age (two weeks). They were housed in groups of three to 8

[1] This investigation was supported by grants from the Commonwealth Fund and the National Vitamin Foundation.

animals in metal wire cages with raised screen bottoms. Food and water were offered ad libitum to all but control animals employed for paired feeding. The food, in the form of a moist paste, was placed in small cups. In order to minimize evaporation and splashing, the drinking water was supplied

TABLE 1

Composition of diets used

BASAL MIXTURE				VITAMIN SUPPLEMENTS [1]	
Component	Diet			Added daily to the diet in mg per 100 gm of basal mixture	
	1	2	3		
	%	%	%		
Sucrose	75	75	75	Thiamine	0.2
Fibrin	18			Riboflavin	0.3
Casein [2]		18	18	Nicotinic acid	2.5
Corn oil [3]	3	3		Ca pantothenate	2.0
Cottonseed hydrogenated oil [4]			3	Inositol	10.0
				Choline chloride	100.0
Salt mixture [5]	4	4	4	Biotin	0.002
				2-Methyl naphthoquinone	1.0
				Given as two drops per week	
				Fat-soluble vitamins A, D and E [6]	

[1] Provided in the form of halibut liver oil diluted 1:2 with corn oil and fed at a level of two drops per week, with α-tocopherol included at 0.5 mg per drop.

[2] Vitamin test casein, General Biochemicals, Inc.

[3] Mazola.

[4] Crisco.

[5] No. 2, U. S. P. XII.

[6] The vitamins were synthetic products of the following manufacturers: thiamine, calcium pantothenate, inositol, biotin, riboflavin, nicotinic acid, choline chloride, General Biochemicals, Inc.; 2-methyl-naphthoquinone (Menadione), Ely Lilly Co.; and α-tocopherol, General Biochemicals, Inc.

from curved graduated glass tubes with a closed top and a turned-up small drinking well at the lower end. The detailed composition of the diets used is presented in table 1. These diets were based on those described by Sarma, Snell and Elvehjem ('46). The hamsters were divided into experimental and control groups. The former groups received no

vitamin B_6, while the control groups were given daily subcutaneous injections of vitamin B_6. All the animals were weighed at least once a week. Food and water intake were recorded for each cage 5 to 6 times a week, and for individual animals when kept singly in metabolism cages for the purpose of collecting urine specimens. The animals were observed for a period of 12 to 13 weeks, unless otherwise stated. At the end of the experiment the survivors were killed by inhalation of chloroform. Tissues of autopsied animals were fixed in Bouin's fluid for routine histological sections and stained with Mayer's hematoxylin and eosin. Some tissues were fixed in 20% formalin in preparation for frozen sections. Xanthurenic acid determinations were made on urine specimens collected in metabolism cages according to the method described by Porter, Clark and Silber ('47), using the Coleman Universal Spectrophotometer Model II and a cuvette 13 mm in diameter. The optical density, corrected for the blank value, and multiplied by 3.9 gave xanthurenic acid concentration in milligrams.

Preliminary observations

These were made on 6 hamsters with initial weights ranging from 46 to 68 gm, divided into experimental and control groups of three each and fed diet 1. The animals of the control group were given daily subcutaneous injections of pyridoxine HCl, 50 μg per dose. The age of the animals used was not exactly known. It was estimated on the basis of weight to be from three to 4 weeks. There was only moderate retardation of growth, and the appearance of increased amounts of xanthurenic acid in the urine. Autopsy performed after three months of observation showed a striking reduction in the size of the lymphoid tissues, notably the thymus, in the deficient animals. Assuming that the age of the animal may be related to ability to develop the deficiency, the following experiments were carried out on two-week-old weanlings.

Vitamin B_6 deficiency in hamsters receiving different types of fat in the diet

Twelve weanling hamsters were divided into 4 groups of three each. Groups 1 and 2 received diet 2, groups 3 and 4, diet 3. The hamsters in groups 3 and 4 received daily subcutaneous injections of 50 μg of pyridoxine HCl.

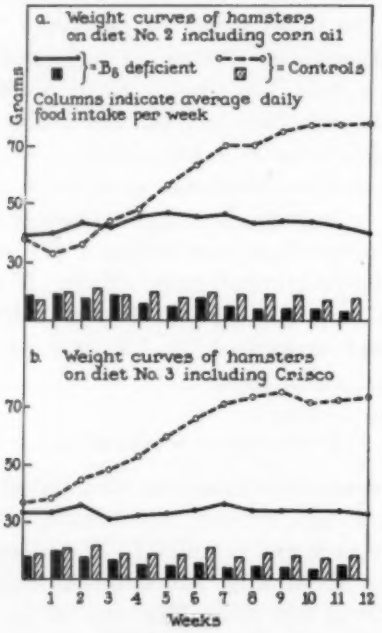
a. Weight curves of hamsters on diet No. 2 including corn oil
= B6 deficient
= Controls
Columns indicate average daily food intake per week
Grams
70
50
30
b. Weight curves of hamsters on diet No. 3 including Crisco
70
50
30
1 2 3 4 5 6 7 8 9 10 11 12
Weeks

Figure 1

As may be seen from figure 1, most of the animals gained weight during the first two weeks. Later the weight curves began to diverge rapidly. The weights of deficient hamsters on commercial hydrogenated cottonseed oil [2] (diet 3) remained almost stationary, occasionally falling below the initial weight. Those on corn oil (diet 2) showed a slightly

[2] Crisco.

better initial gain. After the 5th week, however, the weights of the corn oil animals declined steadily, although remaining at a persistently higher level than those of the hydrogenated cottonseed oil group. A reduction in food intake took place in the deficient animals after the third week (fig. 1), going hand in hand with the weight curve. When food intake was expressed in relation to body weight it appeared that diminished intake resulted not only from loss of appetite but from diminished body size and possibly from a lowered metabolic rate. The deficient animals also showed reduction in water intake ranging up to 50% and a corresponding decrease in urinary excretion. This was not influenced by the fat composition of the diet. There was no evidence of water retention. Xanthurenic acid excretion by the deficient animals rose from a low of 22 mg in the 4th week to a high of 306 mg per 100 gm of ingested protein in the 8th week. In the controls, the amounts were usually from 10 to 15 mg but in one instance, as high as 30 mg. After a short depletion period the animals began to show very striking deviations from normal development. The symptoms varied somewhat in the individual animals, but there were clear-cut differences between the B_6 deficient hydrogenated cottonseed oil and corn oil groups, particularly with respect to the time of onset of deficiency symptoms.

Two animals of group 1 (hydrogenated oil) rapidly developed other signs of deficiency following arrest of growth, in the second and third week, respectively. The velvety texture of the fur changed to a lustreless gray-brown color, with the ends of the hairs sticking to each other as though they were wet or greasy. There was migrating alopecia, mainly about the head and occasionally on the trunk, the rest of the fur becoming thin and lustreless. The nutritional status rapidly deteriorated in the entire group, with disappearance of fat deposits and progressive muscular weakness. The animals became sluggish and developed a tendency to stay motionless, in a hunched position, sleeping a good deal more than the controls. Some malnourished animals appeared to have a

low body temperature, although this was not measured. They survived for weeks in this condition, not growing and consuming very little food. At the end of 6 weeks one animal exhibited a peculiar dragging gait, one showed spreading of the toes of one foot, and another one had paresis of the right foreleg, persisting until death. Growth retardation remained the only sign of B_6 deficiency in group 2 (corn oil) for 12 weeks. Later a rather striking deterioration set in, characterized by considerable weight loss, poor grooming and lack of lustre of the fur, without alopecia. One hamster had an unsteady, fumbling gait, muscular weakness and spreading of the toes. Two animals succumbed in the 14th week.

Post mortem examination at the end of the experiment confirmed the observations made during life. In addition, reduction in size of the lymphoid tissue was most striking. The degree of involution of the thymus varied somewhat in individual animals. In some animals it could be detected only under the microscope (figs. 6 and 7); the residual gland consisted of tiny lobules of lymphoid tissue embedded in atrophic fat tissue. The cortex disappeared and the lymphocytes were reduced in number, resulting in a relative preponderance of the reticular and vascular structures. Even when the involution of the thymus was less pronounced, diminution in the number of lymphocytes was evident. The lymph-nodes showed a reduction in size and a decrease in the number of lymphocytes and secondary follicles. There were similar changes in the Peyer's patches. The number of lymphocytes in the Malpighian follicles of the spleen was moderately reduced. In the testes there was arrested maturation of spermatozoa, although varying degrees of mitosis were present in the majority of seminiferous tubules. All the control animals showed good growth and development and had at all times a lush brown, soft and abundant fur. Their behavior was marked by liveliness. At three months the weight was about three to 4 times that of the deficient animals (fig. 5). The thymuses were well preserved (fig. 8), with abundant lymphoid tissue in the cortex. They weighed

from 17 to 20 mg. The lymph-nodes were easily identified, the peripheral nodes measuring 1 to 2 mm in diameter. The testes contained mature spermatozoa.

Paired feeding and treatment of deficient hamsters with pyridoxine and corn oil

Twenty-four hamsters were divided into three groups of 8 each. Their weights varied from 16 to 36 gm. The average weights of groups 1 and 2 (experimental groups) approximately equalled the average weight of group 3 (control group). Diet 3 containing cottonseed oil as the fat was employed. Pyridoxine HCl, in daily doses of 50 μg, was given only to the hamsters of group 3, beginning with the 5th day of the experiment.

Four control animals of group 3 were started on paired feeding as soon as a reduced ad libitum food intake was noted in the B_6 deficient hamsters; namely, by the end of the second week. Their food intake was regulated so as to keep the weight at a level with that of the deficient animals. They were smaller and thinner than normally but not emaciated, and preserved a normal fur. They were active, retained good muscle tone, and merely appeared younger than their mates fed ad libitum. One control hamster, dead after 9 days of paired feeding, showed no atrophy of the lymphoid tissues. Another one, dead after 4 weeks of paired-feeding, revealed moderate atrophy of the lymph-nodes and thymus. More severe lymphoid atrophy was found in a third one dead after 10 weeks of paired feeding. The 4th one, sacrificed at the same time, showed only a slight reduction in the size of the thymus and lymph-nodes. Fatty changes of the liver were found in all the pair-fed controls.

Treatment was begun after 9½ weeks of the deficient regime, and its effect observed for 3½ weeks. Five hamsters of the group including the larger animals were continued untreated and on the usual diet. The remaining animals were treated with the following substances: pyridoxine HCl alone

(group a); pyridoxine HCl and corn oil (group b); and corn oil alone (group c). Pyridoxine HCl was given in daily doses of 50 μg, by subcutaneous injection. Corn oil was given orally, replacing the hydrogenated fat in the diet.

The weight curves of the deficient and control animals prior to treatment are shown in figure 2; those obtained during the subsequent period of treatment, in figure 3.

The effect of treatment on weight was most striking in group a. The results with pyridoxine and corn oil were less gratifying, possibly because the animals of group b were smaller than those of group a and appeared to suffer from more advanced nutritional deterioration and fur changes

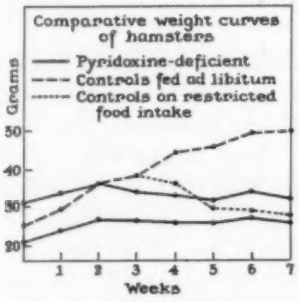

Figure 2

when treatment was begun. Also, they had weighed under 20 gm at weanling age, and developed signs of deficiency more rapidly than the larger weanlings. It is possible that the changes were irreversible and no longer dependent on the absence of pyridoxine. Group c continued to do poorly, showing no response to corn oil. One hamster, which inadvertently had received a single dose of 50 μg of pyridoxine HCl, increased in weight from 24 to 30 gm in less than two weeks. This growth-stimulating effect of pyridoxine did not last beyond two weeks. The average daily food intake indicated that in the deficient animals the intake per 100 gm of body weight diminished as the deficiency progressed. Upon institu-

tion of treatment the food consumption increased, most strikingly in group a. It should be noted that food consumption per 100 gm of body weight was higher in the animals treated with pyridoxine after a depletion period than in those which received this vitamin from the start of the experiment. This suggested a metabolism-stimulating effect of pyridoxine during the recovery and active growth period. Under treatment with pyridoxine there was a reduction of 50% or more in the amount of xanthurenic acid in the urine. Corn oil alone had no effect on the excretion of this substance.

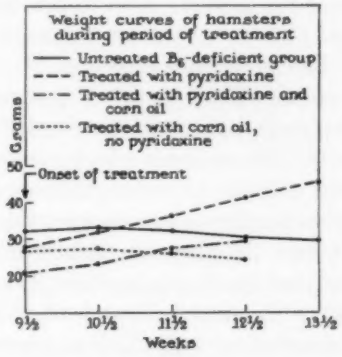

Figure 3

Prior to treatment, growth retardation, fur changes, nutritional deterioration and other clinical signs of deficiency were comparable to those described in the previous experiment. Priapism was noted in two animals about the 6th week, persisting only in one. Some animals showed slight reddening of the skin of the snout or crusting of the upper lip (fig. 10). The animals of group a made a prompt clinical recovery under treatment with pyridoxine. The fur became normal and lustrous within one week, and seemed to increase in amount. The state of nutrition became excellent within a short time. They became more lively and aggressive and their movements recovered quickness and resiliency. They did not

differ from the control animals except for their slightly smaller size (fig. 9). At autopsy there was evidence of regeneration of lymphoid tissue, which was particularly marked in the thymus. The epiphyses of the long bones such as the femur, which showed complete arrest of new bone formation in the deficient hamsters, revealed striking activity, with innumerable newly formed bone trabeculae. In the testes active spermatogenesis was found. In group b, only one hamster showed clinical improvement. The other two persisted in a state of very poor nutrition, although desquamation of the skin on the leg of one cleared up promptly. There was less striking atrophy of the thymus than in the untreated animals, and microscopically there was some evidence of regeneration of lymphocytes. The substitution of corn oil for hydrogenated oil in the diet had no effect on the symptoms of pyridoxine deficiency, the animals of group c resembling the deficient ones in all respects. The animals which received no treatment showed, at the end of 12 to 13 weeks, clinical and post mortem findings of pyridoxine deficiency comparable to those in the previous experiment. It may be mentioned that whenever there was diminished food intake fatty changes were found in the liver, apparently quite independent of the B_6 deficiency.

Comparative anatomical and clinical observations in pair-fed and vitamin B_6 deficient treated and untreated hamsters indicated that the effect of reduced food intake is at least partly responsible for the changes observed in vitamin B_6 deficiency; namely, reduction of fat tissue, atrophy of lymphoid tissue, retardation of skeletal growth and of sexual maturation, and fatty changes in the liver. The alterations of the fur and skin and cachexia, on the other hand, appeared to be independent of the amount of food consumed and directly related to vitamin B_6 deficiency, as these changes responded promptly to treatment with pyridoxine. Moreover, there seemed to be marked alteration of certain metabolic functions of the hamster as manifested by changes in appetite, thirst, excretion of xanthurenic acid, and activity. All these alterations responded to administration of pyridoxine. Corn oil

had no curative action once a vitamin B_6 deficiency had established itself, nor did it promote the therapeutic activity of pyridoxine.

DISCUSSION

The only observation recorded in the literature on vitamin B_6 deficiency in the hamster, by Routh and Houchin ('42), seems to have aroused some controversy. The dermatitis described may have been due to biotin deficiency, as pointed out by Cooperman et al. ('43), who were able to prevent similar mouth lesions by the inclusion in the diet of 1 μg of biotin per day. On the other hand, Hamilton and Hogan ('44) doubted the need of the hamster for biotin as well as for *p*-aminobenzoic acid. It is possible that the requirements are influenced by the composition of the basal diet and by the relative amounts of other vitamins. Although our purified diet included only 2 μg of biotin per 100 gm of diet, no lesions referable to biotin deficiency were observed by us. The mouth lesions occasionally seen were a late manifestation. Prolonged survival of our hamsters also would seem to provide evidence against biotin deficiency, which brings about death in three to 4 weeks (Cooperman et al., '43). The failure to include *p*-aminobenzoic acid and folic acid in our diets did not appear to prevent normal growth and development in the controls; however, the role of these two substances in the nutrition of the hamster requires further study. It should be mentioned also that the amounts of some of the B vitamins used by us were smaller than those employed by Cooperman et al. ('43) and by Hamilton and Hogan ('44). Furthermore, these vitamins were administered in the food, thus allowing a reduced vitamin intake secondary to diminished food consumption. Although the minimum requirements of the hamster for all the vitamins of the B complex are not known, the possibility of multiple deficiencies superimposed on pyridoxine deficiency cannot be completely disregarded.

The syndrome of pyridoxine deficiency in the hamster differs in some respects from that observed in the rat, both

in our experiments with the same diet as used for the hamster and in those published in the literature. While the rate of growth is delayed on a purified diet containing 18% protein, the rat does not show the complete and early arrest of development and the emaciation observed in the hamster under the experimental conditions described. Loss of appetite is one of the earliest and most constant results of pyridoxine deprivation in both species. In our observations, diminution of water intake and urine output was more marked and consistent in the rat than in the hamster. Since the urine of the normal rat contains no xanthurenic acid, in contrast to that of the hamster which usually contains small amounts of this substance, the effect of vitamin B_6 deficiency on xanthurenic acid excretion is more clear-cut in the rat than in the hamster. The most marked divergence in the effect of pyridoxine deficiency between the rat and the hamster is manifest with respect to cutaneous changes. The hamster shows nothing comparable to the classical "rat-acrodynia" first described by György ('34). It may be mentioned in this connection that in our experiments we were unable to produce dermatitis in rats fed a purified diet containing 18% protein and 3% corn oil. When hydrogenated vegetable fat was substituted for corn oil, acrodynia usually appeared about the end of the second month, but did not become as pronounced as has been described by some authors (Sullivan and Nicholls, '40; Antopol and Unna, '42). Severe acrodynia appeared early (after about three weeks) in our experiments with rats on diets containing 30% and 60% protein. Since the need for pyridoxine increases when a high protein diet is given (Miller and Baumann, '45), our observation is not surprising. It remains to be seen whether, by altering the protein content of the basal diet, more accentuated cutaneous alterations can be brought about in the hamster than those observed thus far.

Mouth lesions and priapism occurred less frequently in the hamster than in the rat, while the fur on the trunk and head on a comparable diet remained unaffected in the rat. Epileptiform seizures described in the rat (Chick, El Sadr and

Worden, '40) did not occur in the pyridoxine-deficient hamsters, but there were variable neurologic symptoms such as an ataxic gait or paresis of one extremity and priapism. A relationship between vitamin B_6 and the metabolism of fatty acids has long been recognized, and, although the mechanism is not yet clear, studies on the albino rat indicate that pyridoxine is essential for the maintenance of normal fat metabolism, and that vitamin B_6 and the essential fatty acids complement each other to some extent. Birch ('38) first established the interdependence of vitamin B_6 and the unsaturated fatty acids for their proper utilization by the rat. According to Gross ('40) the dermal manifestations of vitamin B_6 deficiency and fat deficiency (Burr and Burr syndrome) in the rat are identical, and both vitamin B_6 and essential fatty acids are necessary for their cure. The relationship between essential fatty acids and vitamin B_6 was further confirmed by the studies of Salmon ('40, '41), and Schneider, Steenbock and Platz ('40), and, more recently, by Medes and Keller ('47). The latter authors found that alleviation of symptoms of advanced pyridoxine deficiency in rats was attained by either pyridoxine or ethyl linoleate, while for complete cure the administration of both substances was necessary. A similar relationship between pyridoxine and essential fatty acids was suggested by the fact that the symptoms were delayed in hamsters receiving corn oil as compared with those receiving hydrogenated oil as the dietary fat. On the other hand, corn oil was unable to cure in the hamster pyridoxine deficiency once established, or to prevent it completely. This subject needs further investigation. Quantitative and qualitative analyses of body fat similar to those described by Sherman ('47) and Medes and Keller ('47) may throw some light on this problem.

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adequate amounts of food. Diminished food intake is the first clinical manifestation of vitamin B_6 deprivation in young hamsters. A metabolism-stimulating effect of pyridoxine can be recognized in the pair-fed controls, the latter requiring a larger food intake relative to their body weight than the deficient animals for the maintenance of a comparable body weight. The retarding effect on the weight curve may be considered as a direct result of vitamin B_6 deprivation, at least in its initial stage. As the deficiency progresses, with its ensuing malnutrition, it must be assumed that the stunted growth, as well as weight loss, are at least partly secondary phenomena. Changes in behavior as well as alterations of the fur and skin of the B_6-deficient hamsters were not observed in pair-fed controls, and thus are to be considered as deficiency signs independent of inanition. This also holds for the few neurological manifestations seen in the hamster.

As far as pathological changes are concerned, atrophy of lymphoid tissues, and particularly of the thymus, is the most outstanding finding, similar to results obtained with the rat. It cannot yet be decided, however, whether this is due exclusively to pyridoxine deprivation, since it is known that malnutrition as well as deficiency in other food factors may produce atrophy of lymphoid tissues. Jonson ('09) observed rapid thymus involution in young rabbits when subjected to a starvation regime which was qualitatively adequate. Cramer et al. ('21) found reduction in the size of the lymphoid tissues in rats on a "vitamin B" deficient diet. Griffith and Wade ('39) and Christensen and Griffith ('42) observed involution of the thymus as one of the effects of a low choline diet on young rats. In experiments of Stoerk and Zucker ('44) in which adequate amounts of choline were given to rats, marked thymus atrophy was observed even in partial pyridoxine deficiency when weight loss played no role. Our experimental evidence in this connection is equivocal. It appears that atrophy of lymphoid tissues may well have

been the result of inanition, since it was observed in the pair-fed hamsters as well. On the other hand, pyridoxine-depleted animals showed distinct atrophy of lymphoid tissues, even when their nutritional status was well preserved. The microscopic pathology of thymus involution in the vitamin B_6-deficient rat, as described by Stoerk et al. ('47), corresponds to that observed by us in the B_6-deficient hamster; i.e., disappearance of lymphocytes, in the extreme cases, and survival only of epithelial cells and stroma. This picture was found to differ from that of thymus involution in thiamine-deficient rats, in which the lymphocytes did not disappear. It is our impression that the number of lymphocytes encountered depends on the degree of involution rather than on a specific causative factor. It is not possible, on the basis of anatomical observations alone, to distinguish between involution due to different factors, since the anatomical picture appears to be the same regardless of the mechanism of its production. Additional methods, such as the determination of circulating antibodies (Stoerk and Eisen, '46; Axelrod et al., '47) or the use of vitamin analogues (Mushett et al., '47) may be of value.

The amount of choline (i.e., 1 mg daily) given to hamsters in our experiments seems adequate, according to the work of Cooperman et al. ('43). The probability remains, however, that under certain conditions of stress the requirement for choline may be increased. The work of Engel ('42) indicates an increased need for choline in rats made pyridoxine-deficient over longer periods of time. The histological findings of fatty livers in a large percentage of pyridoxine-deficient hamsters, as well as in pair-fed controls, may be suggestive of some choline deficiency. It remains to be seen whether it can be prevented by choline supplementation. Ad libitum-fed controls and B_6-deficient hamsters treated with pyridoxine showed no fatty changes in their livers; however, since their relative food intake was greater than that of the deficient animals, their choline intake also was proportionately greater.

SUMMARY

Male weanling Syrian hamsters were maintained on a purified diet deficient in vitamin B_6. This led to arrest of growth after a depletion period of two to three weeks, diminished food and water intake, progressive malnutrition, muscular weakness and changes of the fur. Increased quantities of xanthurenic acid were found in the urine. The deficient animals did not survive beyond 12 to 13 weeks. Autopsy showed loss of fat tissue and marked atrophy of the lymphoid tissues, notably the thymus, even when malnutrition was mild. There was arrest of sexual maturation and bone growth. Pair-fed controls receiving 50 μg pyridoxine HCl daily showed arrest of growth and atrophy of lymphoid tissues, but failed to develop fur changes, muscular weakness, or cachexia. Controls fed ad libitum and receiving daily 50 μg of pyridoxine HCl showed good growth and nutrition and normal activity. Their fur was normal and there was no atrophy of the lymphoid tissues.

Upon treatment of the deficient animals with daily injections of 50 μg of pyridoxine HCl after a depletion period of 9½ weeks, there were in most animals resumption of appetite and growth, deposition of fat and general return to normal behavior and appearance. Fur changes were repaired within one week of treatment. A single injection of pyridoxine HCl seemed to bring about similar results, the effect lasting for about two weeks. Although the presence of unsaturated fatty acids in the diet seemed to delay the onset of deficiency symptoms, the addition of corn oil to the diet of animals in an advanced state of depletion did not have any beneficial effect.

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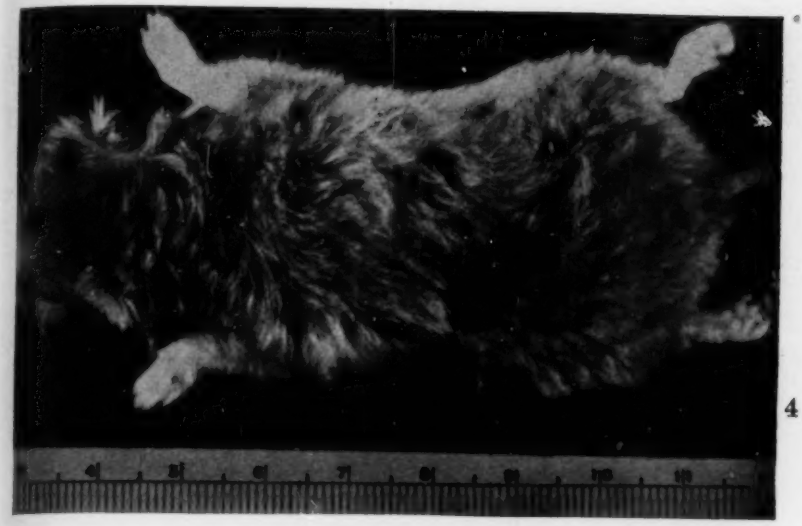
PLATE 1

EXPLANATION OF FIGURES

4 Experiment 2. Hamster, 3½ months old, maintained for three months on vitamin B_6 deficient diet. Note the poor condition of the fur.

5 Experiment 2. A: the same hamster as in figure 4, showing stunted growth. B: Hamster, 3½ months old, which received daily injections of 50 μg of pyridoxine HCl for a period of three months.



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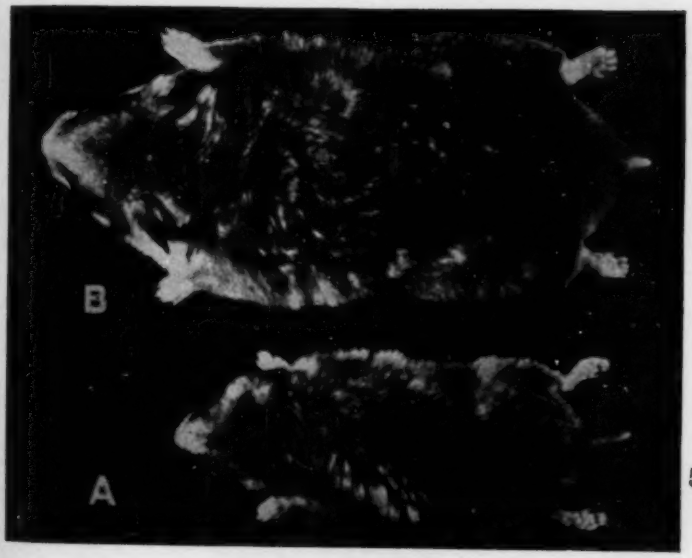

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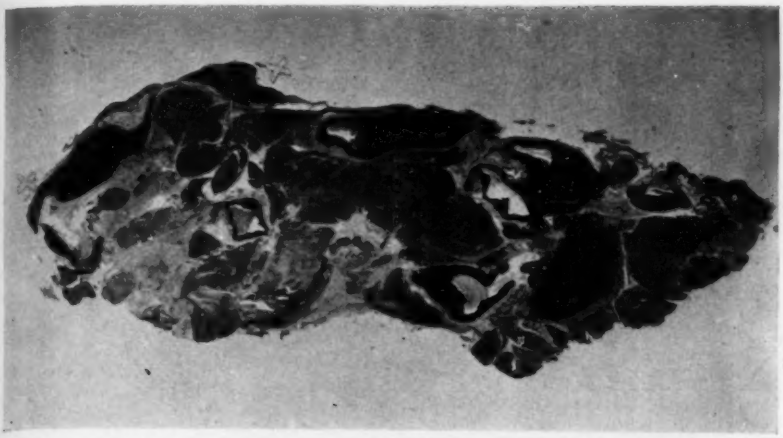
PLATE 2

EXPLANATION OF FIGURES

6 Experiment 2. Thymus of a vitamin B_6-deficient hamster, three months old. The section shows tissue from the anterior mediastinum in which extremely atrophic lobules of thymus are embedded (see *X*). Note also loss of differentiation of cortex and medulla (× 10).

7 Experiment 2. Atrophic thymus of another three-month-old vitamin B_6-deficient hamster, attached to the posterior surface of the sternum (× 10).

8 Experiment 2. Thymus of a hamster, three months old, which received daily injections of 50 μg of pyridoxine HCl for a period of two and one-half months. Note the larger size, and especially the distinct structural differentiation of the thymus (× 10).



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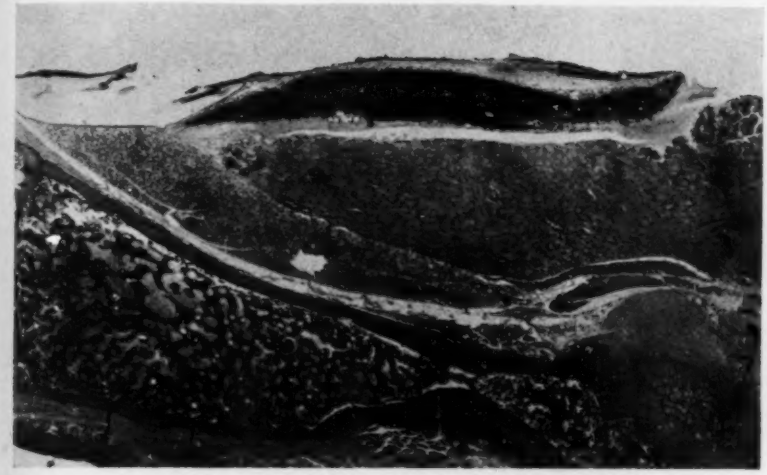

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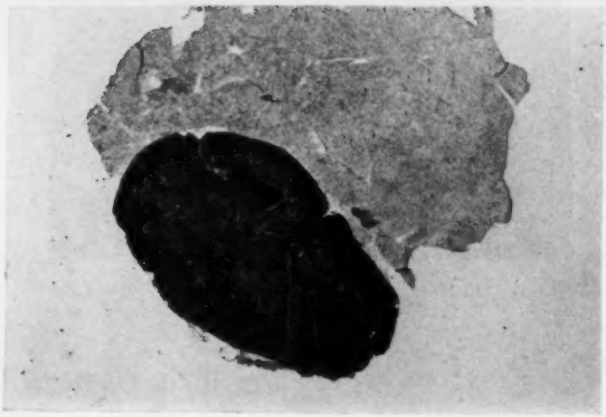

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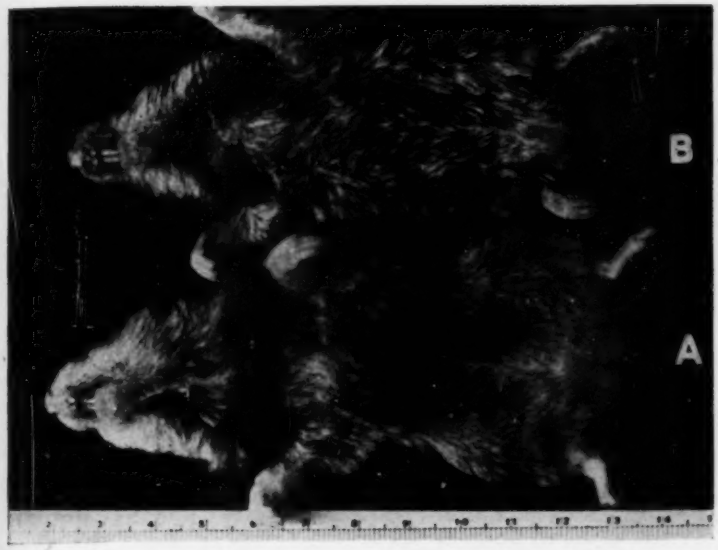
PLATE 3

EXPLANATION OF FIGURES

9 Experiment 3. Effect of treatment with vitamin B_6 on growth, nutritional status and condition of fur. A: hamster depleted of vitamin B_6 for a period of 9½ weeks, then treated with pyridoxine HCl, 50 μg per day for a period of 3½ weeks. B: hamster of the same age, untreated.

10 Experiment 3. Vitamin B_6-deficient hamster showing crusted lesion on upper lip. Photograph taken after a depletion period of 12 weeks.



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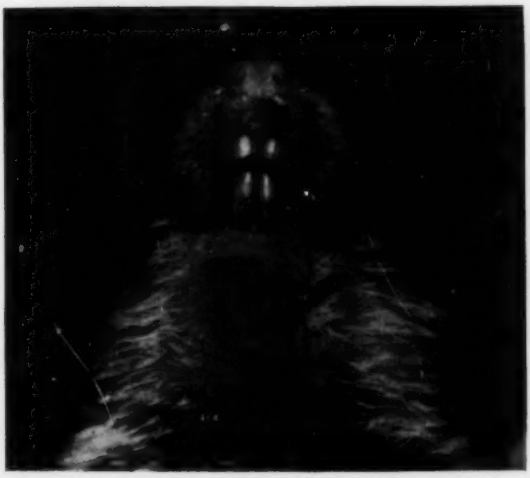

10

URINARY EXCRETION OF AMINO ACIDS AND PEPTIDES BY DOGS FED PROTEIN HYDROLYSATES OR AMINO ACIDS

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When proteins or protein derivatives are administered intravenously, they may or may not be utilized by the animal body. Homologous plasma protein (Holman, Mahoney and Whipple, '34) or hydrolysates of protein have biological value after parenteral administration, whereas heterologous plasma protein (Pommerenke, Slavin, Kariher and Whipple, '35) and gelatin (Jacobsen and Smyth, '44; Brunschwig, Nichols and Bigelow, '46) are of less nutritional value under similar conditions. A large fraction of intravenously administered gelatin is excreted in the urine, showing that such materials must be digested before they can be assimilated by the tissues.

When protein hydrolysates are administered by vein a small portion of the amino acids appears in the urine, and the fraction excreted increases as the total dose is increased. Peptides are also lost in the urine (Cox and Miller, '46; Christensen, Lynch and Powers, '46; Frost and Risser, '46). Mixtures containing DL-amino acids are not as well retained as those composed of L-amino acids (Silber, Seeler and Howe, '46).

The present investigation carried out with dogs deals with the urinary loss of amino acids and peptides after intravenous or oral administration of 6 protein hydrolysates and amino

acid mixtures, all which have been employed for the intravenous alimentation of man. Biological values, expressed here as per cent of the total nitrogen retained, have been determined by a procedure devised in collaboration with a U.S.P. subcommittee headed by Dr. J. B. Allison and Dr. D. V. Frost.

EXPERIMENTAL

The principles of the nitrogen balance procedure used and the calculations based on them have been described in detail by Allison, Seeley and Ferguson ('47). In such studies, whole egg protein and lactalbumin have a nitrogen balance index of 1.0. In other words, when either of these proteins is fed as the sole source of protein nitrogen to dogs excreting about 100 mg N per kilogram per day in the urine, all of the administered nitrogen is retained in the body. Allison et al. fed whole egg protein during three-day control periods between 4-day tests, whereas in the U.S.P. collaborative study lactalbumin was fed during the control periods. In the present studies nitrogen balance data are expressed in terms of per cent nitrogen retained rather than in nitrogen balance indices, which merely involves a shift in decimal point if the basal urinary nitrogen is assumed to represent endogenous nitrogen.

Adult mongrel dogs, which had been fed a protein-free diet (Silber et al., '46) supplemented with lactalbumin (70 cal. and 120 mg N per kilogram per day) for two to three weeks, were fed or infused intravenously with a protein hydroylsate or amino acid mixture daily for a period of 4 days (120 mg N per kilogram per day). During this period lactalbumin was withheld from the diet. In the oral experiments the solutions were given by stomach tube and in the intravenous experiments a constant rate of infusion (2 mg N per kilogram per minute) was maintained by means of an adjustable rate infusion pump. The dogs were dosed by stomach tube with 350 ml of water per 10 kg body weight at the beginning and again at the end of each 4-day period to facilitate quantitative collection of the urines.

The 6 preparations and the urines, collected under toluene, were assayed for α-amino nitrogen by the ninhydrin method (Van Slyke, MacFadyen and Hamilton, '43) before and after acid hydrolysis (6 N HCl, 120°C., 6 hr.). When the dogs received lactalbumin the urinary excretion of free α-amino nitrogen averaged 2.3 mg N per kilogram per day and the

TABLE 1

Composition of preparations

AMINO ACID	PREPARATION					
	A	B	C	D	E	F
	mg/ml					
Arginine	2.2	2.2	1.3	1.5	2.2	3.0
Histidine	0.9	0.9	1.0	1.0	2.4	2.6
Isoleucine	1.4	1.5	2.3	2.2	7.2	6.6
Leucine	4.4	3.1	4.2	4.1	14.9	10.8
Lysine	2.7	2.4	2.5	2.9	10.0	7.5
Methionine	0.5	1.1	1.6	1.1	4.1	2.9
Phenylalanine	2.0	1.7	1.5	2.3	3.9	4.4
Threonine	2.1	2.3	1.6	1.7	1.3	4.2
Tryptophan	0.5	0.3	0.4	0.4	0.8	0.5
Valine	2.4	1.4	2.3	2.4	6.5	8.0
Total N, mg/ml [1]	6.8	7.1	6.5	8.0	13.2	12.8
Free α-amino N, mg/ml [2]	3.1	3.3	2.6	4.0	9.5	10.2
Total α-amino N, mg/ml [2]	5.0	4.7	4.7	5.4	9.2	9.9
Peptides, %	37.6	30.0	44.5	25.0	0.0	0.0

[1] Kjeldahl method.

[2] Free and total α-amino N by gasometric ninhydrin method before and after acid hydrolysis.

bound α-amino nitrogen 1.9 mg N per kilogram per day. These values were determined for each dog prior to infusing or dosing, and the values obtained during the test periods corrected by these amounts. Dogs fed protein-free diets have been found to excrete approximately the same amounts of amino acids, and animals fed stock diets excrete little more (Silber et al., '46).

The Kjeldahl total nitrogen, free α-amino nitrogen, total (free plus peptide) α-amino nitrogen, and essential amino acid contents of the 6 preparations are listed in table 1. The peptide content is expressed as the per cent of the total α-amino nitrogen liberated by acid hydrolysis, and the amino acid analyses were carried out by the methods of Stokes, Gunness, Dwyer and Caswell ('45) without additional hydrolysis.

RESULTS

Oral experiments

When the preparations were administered by mouth, small, almost negligible, fractions of the amino acids were found in the urine, but the excretion of bound amino acids, or peptides, was significantly increased when materials containing peptides were administered. An average of 5.7% of the orally administered peptides was apparently absorbed from the intestinal tract and excreted in the urine. The excretion of amino acids and peptides by two dogs fed each preparation is summarized in table 2. The biological values obtained in nitrogen balance studies, using three or 4 dogs, are also included.

Intravenous experiments

After intravenous infusion at the rate of 2 mg N per kilogram per minute, larger fractions of both amino acids and peptides were excreted (table 2). In general, the loss of peptides in the urine was greater than the loss of free amino acids. This was particularly striking after infusion of preparation C, which gave the lowest value in nitrogen balance experiments.

The effect of rate and route of administration on excretion and biological value

From table 2 it is clear that the biological value of most preparations was about 25% less when given intravenously

than when given orally. This can be correlated with the urinary loss of approximately 15 or 20% of the infused amino acids and peptides.

It has been suggested (Elman and Weiner, '39) that rapid infusion of protein hydrolysates would be impractical due to the probability of greater urinary loss of amino acids. This

TABLE 2

Urinary loss of amino acids and peptides

PREPARATION	FREE α-AMINO N EXCRETED		BOUND α-AMINO N [1] EXCRETED		TOTAL α-AMINO N [2] EXCRETED		TOTAL N RETAINED [3]
	%		%		%		%
			After oral administration				
A	1.6	1.3	3.9	5.6	2.4	2.4	61 ± 5.8
B	1.4	1.6	4.3	6.6	2.2	3.1	64 ± 4.8
C	2.6	2.7	2.6	1.5	2.6	2.1	66 ± 2.0
D	4.4	4.2	6.5	5.5	12.6	8.3	67 ± 3.9
E	1.4	0.3	..		1.0	0.1	73 ± 9.1
F	0.4	0.1	..		0.8	0.0	61 ± 4.0
			After intravenous infusion				
A	12.5	10.7	31.1	29.9	19.5	17.9	45 ± 4.0
B	21.9	18.9	22.6	16.6	24.3	11.3	55 ± 1.0
C	12.9	11.0	46.0	44.6	27.8	25.7	0 ± 13.0
D	8.5	12.3	14.4	14.5	32.3	19.0	40 ± 1.0
E	7.5	3.0	..		8.8	3.2	57 ± 5.0
F	6.6	7.1	..		8.1	8.2	43 ± 6.0

[1] Alpha-amino N after hydrolysis minus α-amino N before hydrolysis.

[2] Alpha-amino N after hydrolysis.

[3] Relative to lactalbumin as 100%; Labco casein gave values of 70, 72, 73, 76, 76, 76, and 78%.

hypothesis was investigated by infusing preparation E or a mixture of peptide-containing hydrolysates (2A + 1C + 1D) into two dogs at 4 times the rate employed in the previous experiment, and comparing the results with those obtained after administration of these materials to the same dogs both orally and intravenously at the slower rate (table 3). When the daily allotment of these preparations was administered

intravenously in only 15 min. nitrogen retention decreased strikingly, but not as a result of excessive loss of amino acid nitrogen in the urine. The retention of nitrogen decreased from about 60% after oral feeding to about 25% after rapid intravenous feeding, although the excretion of amino acids did not increase more than 15%.

The excretion of peptides after oral feeding was again observed with about 10% passing through the body, presumably unchanged. After intravenous infusion 40% of the peptides could be detected in the urine whether the hydrolysate mixture was given over a one-hour or 15-min. period.

TABLE 3

Urinary loss of amino acids and peptides after administration of a composite of preparations A, C, and D or preparation E

PREPARATION	ROUTE	PERIOD OF INFUSION	FREE AMINO ACIDS EXCRETED		PEPTIDES EXCRETED		TOTAL N RETAINED[1]	
			%		%		%	
ACD	Oral	..	0.0	0.0	12.7	8.6	58	62
ACD	I.V.[2]	1 hr.	4.7	5.6	37.9	42.1	40	35
ACD	I.V.	15 min.	14.4	9.4	42.9	40.5	22	32
E	Oral	..	1.4	0.3	...	...	76	56
E	I.V.	1 hr.	7.5	3.0	...	...	49	65
E	I.V.	15 min.	12.6	16.9	...	...	28	22

[1] Relative to oral lactalbumin as 100%.

[2] Intravenous.

DISCUSSION

The 6 preparations were approximately equal to casein in biological value when administered orally to dogs, and about 3% of each preparation was excreted in the urine. After intravenous infusion at a relatively slow rate (2 mg N per kilogram per minute) the retention of nitrogen decreased about 25% and the increased loss of nitrogen (Kjeldahl) in the urine could be ascribed largely to the excretion of amino acids and peptides. A 4-fold increase in the rate of infusion caused a decrease of approximately 60% in biological value, or nitrogen retention. The decreased retention of nitrogen,

however, could not be directly related to an increased loss of amino acids, as suggested by Elman and Weiner ('39). Thus, under the present experimental conditions, infusion of the total nitrogen intake in 15 min. was undesirable due to the fact that only 25% of the administered nitrogen could be utilized.

Loss of free amino acids in the urine varied between 5 and 20% when the preparations were infused at a constant rate for one hour. The reason for this variation is not clear, since the retention of total nitrogen could not be correlated with the loss of amino acids in the urine. It appears that the amino acids of certain preparations are more readily excreted unchanged, whereas those of other preparations are more readily deaminated so that more of their nitrogen is lost as urea and ammonia nitrogen. That the "spillage" of amino acids may be related to the biological value of a preparation, as shown for proteins by Pearce, Sauberlich and Baumann ('47), is indicated by an unpublished experiment in which a tryptophan-free amino acid mixture was administered to dogs. When administered orally to two dogs for 4 days, 40% of the nitrogen was retained in the body, but when given by vein all of the nitrogen was lost in the urine. Seventeen per cent of the amino acids was excreted in the urine after infusion of this preparation, although only 5% was lost when tryptophan was included.

The urinary loss of 30% of the peptides infused shows that the peptides of the hydrolysates were not as well retained as the amino acids, which were lost in the urine to the extent of 14%. The fact that increasing the rate of infusion 4-fold did not increase the peptide excretion suggests that the greater loss of peptides in the urine was not solely a result of inability of the kidney tubules to reabsorb peptides from the glomerular filtrate. An alternative explanation is that certain of these peptides were not available to the tissues, due either to their size or composition.

The incomplete utilization of parenterally administered peptides (Christensen et al., '46) is most clearly demonstrated

by the experiments with preparation C. When this hydrolysate was given intravenously to 4 dogs about half of the peptide fraction was found in the urine and the biological value was zero—that is, none of the nitrogen was retained by the body. However, when the same preparation was given orally and thereby exposed to intestinal digestion, it was utilized as well as the other preparations. It appears that certain essential amino acids may have been primarily in the peptide fraction that was lost in the urine after intravenous infusion. This would obviously make the preparation deficient although all essential amino acids were present in the material infused. It is also a possibility that hydrolysate C satisfied the requirements for oral administration to dogs but not those for intravenous administration. We have no reason to assume that the relative amounts of amino acids required in the diet are the same as those required for parenteral feeding. It is not unreasonable to assume that certain amino acids may be utilized less efficiently than others by the intravenous route, and might therefore be required in relatively higher concentrations when the parenteral route is employed.

The data emphasize the necessity of demonstrating that preparations designed for intravenous alimentation can be utilized after parenteral infusion. However, it does not follow that a preparation which is not satisfactorily utilized by laboratory animals, under a given set of conditions, will necessarily be unsatisfactory in man.

Further evidence has been obtained of the unavailability of certain peptides after oral feeding of protein hydrolysates. In these experiments, after feeding three hydrolysates or a mixture of the same hydrolysates, 5 to 10% of the ingested peptides were found in the urine. Since there was little or no increase in peptide amino nitrogen in the urine after the feeding of preparations containing no peptides (E and F) it is reasonable to conclude that the bound α-amino nitrogen found after feeding peptides was actually peptide in nature, and had its origin in the peptide component of the hydrolysate.

SUMMARY

1. The urinary excretion of amino acids by dogs was negligible during a 4-day period of oral feeding of protein hydrolysates or amino acid mixtures. After intravenous administration over a period of one hour per day, the loss of free amino acids in the urine ranged from 5 to 20% and there was a decrease in nitrogen retention of approximately 25%. When the daily allotment was given by vein in only 15 min., the loss of amino acids in the urine increased 10 to 15% and the utilization of nitrogen decreased approximately 60%, due to greater excretion of urea and ammonia nitrogen.

2. After oral feeding of preparations containing peptides, 2 to 11% of the peptide fraction was lost in the urine. After infusion by vein during a one-hour period an average of 30% of the peptides was excreted, in contrast to only 14% of the free amino acids of these preparations. A 4-fold increase in the rate of infusion resulted in no additional loss of peptides in the urine.

3. One preparation, which was utilized approximately as well as casein when administered orally, was found to have no biological value in terms of nitrogen retention when given intravenously. This was apparently related to the excessive urinary loss of its peptides (45%) and its amino acids (12%).

4. The degree of utilization of protein hydrolysates and amino acid mixtures depends upon the method of feeding and upon the rate of infusion.

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THE EFFECT OF FAT ON CALCIUM AND PHOSPHORUS METABOLISM IN NORMAL GROWING RATS UNDER A NORMAL DIETARY REGIME [1]

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Much work has been done on the effect of fat on calcium and phosphorus metabolism. In most instances either the diet or the animals employed, or both, have been abnormal. The criteria used for assessing results also have varied. Thus Westerlund ('34a, '34b), investigating the influence of tripalmitin, triolein and tributyrin upon calcium metabolism in the adult rat, found that the feeding of tripalmitin caused negative calcium balances while neither triolein nor tributyrin produced such an effect. It is to be noted, however, that the level of calcium fed was kept extremely low to promote the highest degree of calcium utilization. French ('42) and French and Elliott ('43) fed diets containing oleo oil at levels ranging from 0.28% to 45.46% to adult rats. The diets contained the suboptimum level of 0.3% of calcium and a calcium-to-phosphorus ratio of 1:1. They concluded that oleo oil interferes to a slight degree with calcium retention, though the evidence was of a low order of significance.

Knudson and Floody ('40) fed rats a low-fat rachitogenic diet containing a known amount of vitamin D, 1.27% calcium

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and a calcium-to-phosphorus ratio of 5:1. They found that the addition of 5% hardened cottonseed oil promoted better healing in rachitic rats. Larger amounts of fat were not so efficacious. Similarly, Jones ('40) found that the addition to rachitogenic diets of lard to comprise up to 10% increased the bone ash of rachitic rats over a wide range of calcium levels (0.38%–1.0%) and calcium-to-phosphorus ratios (4:1–50:1). Higher levels of lard exerted less influence.

As a result of many studies involving the addition of peanut oil to a high calcium-low phosphorus rachitogenic diet, Booth, Henry and Kon ('42) concluded that fats exert a definite antirachitic effect. They do not exert such an effect in high phosphorus-low calcium diets. Bunkfeldt and Steenbock ('43) fed cottonseed oil as a supplement to various diets and concluded that calcification was depressed in a low-phosphorus rachitogenic ration (phosphorus 0.075%). When phosphorus was optimum (0.25%), calcification was increased. Further increases in phosphorus content indicated a much smaller beneficial effect of fat, which was quite independent of the calcium-to-phosphorus ratio.

While fat most certainly affects the utilization of calcium and phosphorus, it appears that the absolute level of calcium and of phosphorus, their ratio to each other, the fat used and the condition of the animal are important factors. It seemed to us desirable to determine the influence of fat on calcium and phosphorus utilization when a normal diet is fed to normal, growing rats.

EXPERIMENTAL

The percentage composition of the ration used was egg albumen (cooked), 18.0; dextrinized starch, 73.4; and Jones and Foster ('45) salt mixture, 3.6. To this was added 5% of the fat to be used–completely hydrogenated cottonseed oil (HCSO), peanut oil (PNO) or coconut oil (CNO). One group was given an additional 5.2% of dextrinized starch in lieu of fat, the salt mixture being dropped to 3.4% so as to preserve

a constant ratio of calories to minerals. Daily vitamin supplements of 80 μg thiamine hydrochloride, 160 μg riboflavin, 120 μg pyridoxine hydrochloride and 100 μg of calcium pantothenate were given. Vitamins A and D were supplied by one drop of cod liver oil twice each week. The rations provided approximately 0.6% of calcium and 0.4% of phosphorus.[3]

Twelve 60-gm male rats were placed on each diet. In the case of the hydrogenated cottonseed oil, however, the feces and urine collections were from 10 rats, as the urine from two of the animals was inadvertently discarded after the completion of the feeding. The rats were kept in individual mesh-bottomed metabolism cages, each of which was set in a funnel in which had been placed a copper screen for the separation of feces and urine. Collections were started following a two-day preliminary feeding period. Feces were removed from the screen each day and the cage bottom, screen, and funnel washed with hot water acidified with sulfuric acid to a pH of 4.6 to 4.8. Twice each week, the urine, plus washings, was collected from beakers placed under the funnels and stored in glass jars at 7 to 8°C. until analyzed.

Food wastes and contamination with excreta were held to negligible quantities by the use of a specially prepared feeding device. A Franke-type cup was placed in the center of a can having a diameter of about 5 inches, the can being cut off at an angle of approximately 30° and the whole assembly covered with quarter-inch mesh and suspended on the side of the cage. By this arrangement, sleeping on the feeding cup was prevented and scattered food fell through the mesh into the large can, permitting subsequent recovery and weighing.

At the conclusion of a 28-day feeding period, the rats were killed with chloroform and the femurs removed for bone ash determinations. Calcium and phosphorus determinations were made of the diets and samples of the feces and urine.

[3] The actual percentage of calcium and phosphorus was as follows: HCSO diet — Ca 0.68, P 0.42; CNO diet — Ca 0.63, P 0.40; PNO diet — Ca 0.65, P 0.40; no-fat diet — Ca 0.61, P 0.44.

The methods of Morris, Nelson and Palmer ('31) were used in these determinations.

RESULTS

Calcium and phosphorus balances

Data on food intakes, weight gains and calcium and phosphorus determinations are given in table 1. Initially an attempt was made to control rigidly and equalize the amount of food eaten. This did not prove feasible, possibly because of a difference in appetite for the various fats. Sufficient

TABLE 1

Results of chemical determinations and feeding trials

CATEGORY OF INTEREST	DIET			
	HCSO	CNO	PNO	No-fat
Food intake (gm)	297.3	307.7	295.5	311.0
Weight gain (gm)	121.4	115.5	116.5	117.7
Calcium				
In food (gm)	2.0 ± 0.08[1]	1.9 ± 0.04	1.9 ± 0.06	1.9 ± 0.12
In feces (gm)	0.8 ± 0.07	0.7 ± 0.05	0.6 ± 0.08	0.4 ± 0.23
In urine (gm)	0.1 ± 0.07	0.1 ± 0.02	0.2 ± 0.05	0.2 ± 0.12
Phosphorus				
In food (gm)	1.3 ± 0.05	1.2 ± 0.02	1.2 ± 0.08	1.4 ± 0.10
In feces (gm)	0.2 ± 0.02	0.3 ± 0.03	0.3 ± 0.04	0.3 ± 0.08
In urine (gm)	0.2 ± 0.06	0.1 ± 0.04	0.1 ± 0.04	0.1 ± 0.08
Femur ash (%)	61.9 + 1.90	62.8 + 0.70	62.0 + 1.80	62.0 + 1.00

[1] Standard deviation.

control was maintained, however, so that the mean variation of the amounts eaten was not large. All animals gained well.

Calculated values of the proportion of calcium and phosphorus in the feces and urine and that which was retained are given in table 2. The significance of the differences between the means of calcium and phosphorus in the feces and in the urine for each fat and the comparable mean for the no-fat diet were tested, using Fisher's ('30) modification of "Student's" t test.

Fecal calcium increased with increase in the melting point of the fat used. The animals on the no-fat diet excreted the least calcium in the feces. The differences between the means of the HCSO and CNO diets, respectively, and that of the no-fat diet were highly signficant; between the mean of the PNO diet and the no-fat diet, the differences were below the significant level.

In general, urinary calcium varied inversely to fecal calcium, though the variations were of smaller magnitude. Hence, retentions of calcium varied nearly as greatly as the fecal

TABLE 2

Partition of dietary calcium and phosphorus

CATEGORY OF INTEREST	DIET			
	HCSO	CNO	PNO	No-fat
Calcium				
Fecal (%)	42 ± 2.5 [1]	35 ± 3.2 [1]	30 ± 4.5	27 ± 4.4
Urinary (%)	3 ± 0.1 [1]	7 ± 1.9	7 ± 3.1	8 ± 1.8
Retention (%)	55 ± 2.6 [1]	59 ± 2.7 [1]	63 ± 1.7	65 ± 4.3
Phosphorus				
Fecal (%)	18 ± 2.9	27 ± 3.4 [1]	26 ± 4.8 [2]	20 ± 1.8
Urinary (%)	19 ± 2.1 [1]	6 ± 1.7	5 ± 1.8	6 ± 2.1
Retention (%)	63 ± 4.4 [1]	67 ± 4.1 [1]	69 ± 4.9 [2]	74 ± 3.6

[1] The difference between this value and that of the fat-free diet is highly significant ("t" value exceeds that tabulated for $P = 0.01$).

[2] The difference between this value and that of the fat-free diet is significant ("t" value exceeds that tabulated for $P = 0.05$).

calcium, but in inverse order. Again the difference between the means was highly significant in the case of the HCSO and CNO diets, but not significant in the case of the PNO diet.

The excretion of fecal phosphorus revealed no constant relationship either to the characteristics of the fat used or to the presence or absence of fat in the diet. Animals on the CNO and PNO diets excreted more phosphorus than those on the no-fat diet. There was little difference between results on the HCSO and no-fat diets.

The very high excretion of phosphorus in the urine of rats on the HCSO diet was the only noteworthy feature of urinary phosphorus excretion. It lowered the retention of phosphorus to a value less than that obtained on any other diet; consequently retentions of phosphorus varied in the same manner as retentions of calcium. The difference between the means of the HCSO and CNO diets and that of the no-fat diet were highly significant; that between the means of the PNO diet and the no-fat diet was significant.

Excretion of calcium soaps

Holt, Courtney and Fales ('20) suggested that certain instances of poor calcium retention were due to the formation of insoluble calcium soaps. In order to discover the extent

TABLE 3

Neutral fat and fatty acids of feces

CATEGORY OF INTEREST	DIET			
	HCSO	CNO	PNO	No-fat
Dry weight of feces (gm)	25.36	18.22	16.19	17.94
Neutral fat in feces (gm)	2.28	0.58	0.82	0.60
Free fatty acids in feces (gm)	10.64	1.13	1.96	1.44
Total (as triglycerides) (gm)	13.41	1.76	2.86	2.10

to which this reaction might be responsible for the higher fecal calcium noted in rats fed the fat-containing diets, we attempted to determine the quantity of calcium soaps excreted in the feces. Several methods were tried and that of Augur, Rollman and Deuel ('47) was finally used, although it did not prove entirely satisfactory. The results are given in table 3.

Rats on the no-fat diet excreted considerable quantities of neutral fat and soaps. These, as Augur et al. have pointed out, must be regarded as of "metabolic" origin.

The animals on the CNO diet excreted even less neutral fat and soaps; hence it would appear that all of the fat in

their diet was absorbed, since the quantities found in the feces were of such small magnitude that they could all be accounted for as metabolic. This agrees with the findings of Hoagland and Snider ('43a) that coconut oil is 98.9% digestible at the 5% level. The relatively high fecal excretion of calcium noted in rats on this diet cannot be attributed to the formation of calcium soaps.

Rats on the PNO diet excreted slightly larger quantities of neutral fat and soaps than did rats on the no-fat diet.

Particularly noteworthy is the much greater amount of feces passed by animals on the HCSO diet than that by animals on the other diets. All of this increase was accounted for by increases in the amounts of free fat and soap excreted, which was expected in view of the higher fecal excretion of calcium observed.

Bone formation

Data on the analysis of the femurs for ash is given in table 1. It can readily be seen that, at least in the 28 days of feeding, the differences in mineral metabolism obtained on the diets did not effect the formation of the bones.

DISCUSSION

The inclusion of 5% fat in the diet increased fecal calcium. From the work of Holt, Courtney and Fales ('20) one might conclude that the rather large increase in fecal calcium in rats on the HCSO diet was probably due to poor absorption of calcium stearate formed in the digestive tract. Hoagland and Snider ('43b) have also reported on the poor absorption of tristearin and stearic acid. The HCSO was completely hydrogenated, hence it would have contained a large percentage of stearate. The large amounts of free fatty acids (soaps) noted in the feces of these animals would tend to substantiate such a theory.

On the other hand, the excretion of free fatty acids (soaps) by rats on the CNO and PNO diets was much smaller, so that it seems improbable that such a reaction was a factor in the

The methods of Morris, Nelson and Palmer ('31) were used in these determinations.

RESULTS

Calcium and phosphorus balances

Data on food intakes, weight gains and calcium and phosphorus determinations are given in table 1. Initially an attempt was made to control rigidly and equalize the amount of food eaten. This did not prove feasible, possibly because of a difference in appetite for the various fats. Sufficient

TABLE 1

Results of chemical determinations and feeding trials

CATEGORY OF INTEREST	DIET			
	HCSO	CNO	PNO	No-fat
Food intake (gm)	297.3	307.7	295.5	311.0
Weight gain (gm)	121.4	115.5	116.5	117.7
Calcium				
In food (gm)	2.0 ± 0.08 [1]	1.9 ± 0.04	1.9 ± 0.06	1.9 ± 0.12
In feces (gm)	0.8 ± 0.07	0.7 ± 0.05	0.6 ± 0.08	0.4 ± 0.23
In urine (gm)	0.1 ± 0.07	0.1 ± 0.02	0.2 ± 0.05	0.2 ± 0.12
Phosphorus				
In food (gm)	1.3 ± 0.05	1.2 ± 0.02	1.2 ± 0.08	1.4 ± 0.10
In feces (gm)	0.2 ± 0.02	0.3 ± 0.03	0.3 ± 0.04	0.3 ± 0.08
In urine (gm)	0.2 ± 0.06	0.1 ± 0.04	0.1 ± 0.04	0.1 ± 0.08
Femur ash (%)	61.9 + 1.90	62.8 + 0.70	62.0 + 1.80	62.0 + 1.00

[1] Standard deviation.

control was maintained, however, so that the mean variation of the amounts eaten was not large. All animals gained well.

Calculated values of the proportion of calcium and phosphorus in the feces and urine and that which was retained are given in table 2. The significance of the differences between the means of calcium and phosphorus in the feces and in the urine for each fat and the comparable mean for the no-fat diet were tested, using Fisher's ('30) modification of "Student's" t test.

Fecal calcium increased with increase in the melting point of the fat used. The animals on the no-fat diet excreted the least calcium in the feces. The differences between the means of the HCSO and CNO diets, respectively, and that of the no-fat diet were highly signficant; between the mean of the PNO diet and the no-fat diet, the differences were below the significant level.

In general, urinary calcium varied inversely to fecal calcium, though the variations were of smaller magnitude. Hence, retentions of calcium varied nearly as greatly as the fecal

TABLE 2

Partition of dietary calcium and phosphorus

CATEGORY OF INTEREST	DIET			
	HCSO	CNO	PNO	No-fat
Calcium				
Fecal (%)	42 ± 2.5 [1]	35 ± 3.2 [1]	30 ± 4.5	27 ± 4.4
Urinary (%)	3 ± 0.1 [1]	7 ± 1.9	7 ± 3.1	8 ± 1.8
Retention (%)	55 ± 2.6 [1]	59 ± 2.7 [1]	63 ± 1.7	65 ± 4.3
Phosphorus				
Fecal (%)	18 ± 2.9	27 ± 3.4 [1]	26 ± 4.8 [2]	20 ± 1.8
Urinary (%)	19 ± 2.1 [2]	6 ± 1.7	5 ± 1.8	6 ± 2.1
Retention (%)	63 ± 4.4 [1]	67 ± 4.1 [1]	69 ± 4.9 [2]	74 ± 3.6

[1] The difference between this value and that of the fat-free diet is highly significant ("t" value exceeds that tabulated for P = 0.01).

[2] The difference between this value and that of the fat-free diet is significant ("t" value exceeds that tabulated for P = 0.05).

calcium, but in inverse order. Again the difference between the means was highly significant in the case of the HCSO and CNO diets, but not significant in the case of the PNO diet.

The excretion of fecal phosphorus revealed no constant relationship either to the characteristics of the fat used or to the presence or absence of fat in the diet. Animals on the CNO and PNO diets excreted more phosphorus than those on the no-fat diet. There was little difference between results on the HCSO and no-fat diets.

The very high excretion of phosphorus in the urine of rats on the HCSO diet was the only noteworthy feature of urinary phosphorus excretion. It lowered the retention of phosphorus to a value less than that obtained on any other diet; consequently retentions of phosphorus varied in the same manner as retentions of calcium. The difference between the means of the HCSO and CNO diets and that of the no-fat diet were highly significant; that between the means of the PNO diet and the no-fat diet was significant.

Excretion of calcium soaps

Holt, Courtney and Fales ('20) suggested that certain instances of poor calcium retention were due to the formation of insoluble calcium soaps. In order to discover the extent

TABLE 3

Neutral fat and fatty acids of feces

CATEGORY OF INTEREST	DIET			
	HCSO	CNO	PNO	No-fat
Dry weight of feces (gm)	25.36	18.22	16.19	17.94
Neutral fat in feces (gm)	2.28	0.58	0.82	0.60
Free fatty acids in feces (gm)	10.64	1.13	1.96	1.44
Total (as triglycerides) (gm)	13.41	1.76	2.86	2.10

to which this reaction might be responsible for the higher fecal calcium noted in rats fed the fat-containing diets, we attempted to determine the quantity of calcium soaps excreted in the feces. Several methods were tried and that of Augur, Rollman and Deuel ('47) was finally used, although it did not prove entirely satisfactory. The results are given in table 3.

Rats on the no-fat diet excreted considerable quantities of neutral fat and soaps. These, as Augur et al. have pointed out, must be regarded as of "metabolic" origin.

The animals on the CNO diet excreted even less neutral fat and soaps; hence it would appear that all of the fat in

their diet was absorbed, since the quantities found in the feces were of such small magnitude that they could all be accounted for as metabolic. This agrees with the findings of Hoagland and Snider ('43a) that coconut oil is 98.9% digestible at the 5% level. The relatively high fecal excretion of calcium noted in rats on this diet cannot be attributed to the formation of calcium soaps.

Rats on the PNO diet excreted slightly larger quantities of neutral fat and soaps than did rats on the no-fat diet.

Particularly noteworthy is the much greater amount of feces passed by animals on the HCSO diet than that by animals on the other diets. All of this increase was accounted for by increases in the amounts of free fat and soap excreted, which was expected in view of the higher fecal excretion of calcium observed.

Bone formation

Data on the analysis of the femurs for ash is given in table 1. It can readily be seen that, at least in the 28 days of feeding, the differences in mineral metabolism obtained on the diets did not effect the formation of the bones.

DISCUSSION

The inclusion of 5% fat in the diet increased fecal calcium. From the work of Holt, Courtney and Fales ('20) one might conclude that the rather large increase in fecal calcium in rats on the HCSO diet was probably due to poor absorption of calcium stearate formed in the digestive tract. Hoagland and Snider ('43b) have also reported on the poor absorption of tristearin and stearic acid. The HCSO was completely hydrogenated, hence it would have contained a large percentage of stearate. The large amounts of free fatty acids (soaps) noted in the feces of these animals would tend to substantiate such a theory.

On the other hand, the excretion of free fatty acids (soaps) by rats on the CNO and PNO diets was much smaller, so that it seems improbable that such a reaction was a factor in the

increases of fecal calcium noted in these two groups of animals. Calcium stearate is utilized by the rat, to a limited extent at least, as Boyd, Crum and Lyman ('32) have shown. It would seem likely that the much smaller amounts of calcium stearate which might have beeen formed on the CNO and PNO diets were largely absorbed.

Bergeim ('26) demonstrated that calcium is excreted into the intestine. Hence, the presence of calcium in the feces may be due to lack of absorption or to excretion. The extent of excretion varies over a considerable range, depending on the diet. Following a period of feeding calcium citrate, Steggerda and Mitchell ('46) found fecal calcium to be as much as twice the calcium intake. Greenberg ('45) found that about 18.0% of an injected dose of radioactive calcium appeared in the feces, whereas 32.5% appeared when the same amount was administered orally. It was not possible in the present experiments to determine whether the increased calcium in the feces of those rats fed the CNO and PNO diets was due to decreased absorption, to increased excretion, or to both.

It does seem that the increased excretion of fecal calcium imposed no strain on the economy of calcium in the body except, possibly, in those rats on the HCSO diet. Urinary calcium remained nearly constant except for the latter group. Bone ash apparently was not affected in any group, which is interesting in view of the marked variation in retention from group to group.

Phosphorus, too, is excreted into the intestine, as Bergeim ('26) has shown, and its presence in the feces may be due to lack of absorption or to excretion. Though the addition of CNO and PNO to the diet increased fecal phosphorus, HCSO did not have such an effect. This may have been due to the removal of interfering calcium from the intestine, as calcium stearate, when HCSO was fed. With the CNO and PNO diets there was little, if any, increase in soap formation but there was an increase in calcium excretion, possibly as a phosphate, thus accounting for the observed increase in

phosphorus excretion. Certainly there was no indication that the inclusion of fat, per se, increased fecal phosphorus.

Urinary phosphorus, like urinary calcium, was not much affected except in the group on the HCSO diet. Though fecal phosphorus was the lowest in this group, urinary phosphorus was over three times as great as in the other groups. Such an effect might have been due to a higher plasma concentration after absorption, giving rise to an excessive excretion before the phosphorus could be used, or to a lack of calcium ions which would aid in fixing phosphorus in the tissues.

The intimate relationship existing between calcium and phosphorus metabolism was evident in the values obtained for phosphorus retention. In spite of the irregular and divergent effect of the different fats on phosphorus excretion in the feces and urine, its retention varied in a manner similar to that of calcium.

When calcium is in excess, as in many rachitogenic diets, and the fat is partly composed of triglycerides of long-chain, saturated fatty acids, the formation of calcium soaps, with consequent lowering of phosphorus lost in the feces, may bring about some improvement in phosphorus utilization. This may possibly explain the results of Knudson and Floody ('40) and of Jones ('40). The observation of Booth, Henry and Kon ('42) that the equicaloric inclusion of peanut oil in the diet lowered both the fecal excretion of phosphorus and the urinary excretion of calcium is in direct contrast to our results. It was possibly due to the higher calcium and lower phosphorus content of their rachitogenic diet (calcium 0.9%, phosphorus 0.18%) and the formation of calcium soaps when fat was included.

CONCLUSIONS

1. The inclusion in a normal diet of 5% fat (completely hydrogenated cottonseed oil, coconut oil, or peanut oil) increased the fecal excretion of calcium.

2. When fats containing many of the poorly absorbed glycerides of the long-chain, saturated fatty acids were fed,

the increase in fecal excretion was probably due to the formation of relatively insoluble calcium soaps. When this was the case, the excretion of phosphorus in the feces was not increased.

3. When fats containing mostly the more rapidly absorbed glycerides of long-chain, unsaturated or short-chain, saturated fatty acids were fed, the increase in fecal excretion of calcium was of lesser magnitude. It did not seem to be due to the formation of calcium soaps and it apparently carried phosphorus with it, since fecal phosphorus was also increased.

4. The excretion of calcium and phosphorus in the urine was affected only indirectly by the presence of fat in the diet. When the fecal excretion of calcium was considerably increased there was a decrease in the urinary excretion of calcium but a marked increase in the urinary excretion of phosphorus.

5. The cumulative result of the inclusion of 5% fat in the diet was a decrease in the retentions of calcium and of phosphorus which roughly paralleled the increase in the fecal excretion of calcium.

6. The inclusion of 5% fat in the normal diet, well supplied with calcium and phosphorus and having an optimum calcium-to-phosphorus ratio, did not apparently affect the formation of bone in healthy, growing rats.

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PURIFIED AMINO ACIDS AS A SOURCE OF NITROGEN FOR THE GROWING RAT [1]

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More than 10 years ago Rose and co-workers demonstrated that 10 amino acids are required by the rat for rapid growth, while 12 other amino acids are dispensable. An extensive review of this subject appeared in 1938 (Rose, '38). At about the same time Rose ('37) presented tentative minimum requirements for each of the 10 indispensable amino acids for rapid growth of the rat. A reinvestigation by Rose and Womack ('46) when an improved ration was used showed that the phenylalanine requirement proposed earlier (0.7%) was somewhat low, and that 0.9% was required for maximum growth. This requirement was reduced to nearly half when the ration contained about 0.6% L-tyrosine (Womack and Rose, '46b).

Subsequent work by this group (Borman et al., '46) confirmed their earlier observation (Scull and Rose, '30) that the young rat can synthesize arginine from materials ordinarily available in the ration, but not at a rate commensurate with the requirements for rapid growth. Therefore, arginine was classified as an essential dietary component for optimum

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growth. Using an improved ration in which the composition of the amino acid mixture, vitamin supplement, and gross constituents were altered, Womack and Rose ('47) investigated the nutritional role of glutamic acid, hydroxyproline and proline. They found that hydroxproline and proline were dispensable, but that the latter could partially replace the arginine required for rapid growth. Arginine, glutamic acid and proline were mutually interconvertible in the rat but at different rates, as shown by the decreasing order of their influence upon growth. However, the best rates of growth were obtained when all three amino acids were included. Later studies (Rose et al., '48) indicated that glutamic acid was stimulatory when added to a ration containing only the 10 essential amino acids, but its omission from a ration containing 19 amino acids did not reduce the growth rate significantly.

Evidence of the existence in proteins of an unidentified growth stimulant for rats was presented by Womack and Rose ('46a), who observed that the growth rate of animals receiving their best amino acid ration was far less than that of rats receiving a ration containing 18% casein supplemented with 0.2% methionine (3.5 gm per day compared to 5.1 gm per day). Partial replacement of the amino acids by proteins such as casein improved the growth rate while the corresponding acid hydrolysates did not. It was concluded therefore that young rats require an unknown growth factor contained in intact proteins. Whether this is identical with "strepogenin," the peptide which has been reported to be a growth stimulant for certain bacteria and mice (Woolley, '45), was not established.

The present communication deals with studies begun nearly three years ago in an effort to formulate a mixture of purified amino acids which would support growth rates in weanling rats approximating those obtainable with equivalent amounts of a protein such as casein. For this purpose the minimum requirements for the essential amino acids as proposed by

Rose in 1937 and modified subsequently (Womack and Kade, '44) served as a starting point.

EXPERIMENTAL

Weanling male rats of the Sprague-Dawley strain, 21 days old and within a 5 gm weight range, were used. They were placed in individual cages and fed ad libitum for 28 days. In most of the groups the daily food consumption was recorded to permit the calculation of the protein efficiency of the different rations. The animals were weighed at weekly intervals.

Each group consisted of two to 4 animals, with three in a majority of the groups. Whenever a suggestive observation was made the experiment was repeated with a larger number of animals, using the same ration, and the results of all such trials are presented together. The growth rates and protein efficiency values given in the tables represent the averages together with the range or the standard error of the mean. When possible, the "t" test was applied and a P value of 0.05 or less was considered statistically significant.

The rations used had the following basic percentage composition: Corn oil 5, salts IV (Phillips and Hart, '35) 4, monosodium phosphate monohydrate [4] 0.63, vitamin mixture 2, amino acids, casein [5] or acid hydrolysate of casein [6] as indicated, and sucrose to make up to 100%. The 2 gm of the vitamin mixture in a sucrose base contained: Thiamine hydrochloride 0.2 mg, riboflavin 0.3 mg, pyridoxine hydrochloride 0.25 mg, niacin 1.5 mg, inositol 10 mg, choline chloride 100 mg, calcium *d*-pantothenate 2 mg, biotin 10 μg, and pteroylglutamic acid 20 μg. Fat-soluble vitamins were given once each week to each rat in the form of two drops of a modified halibut liver oil to provide a daily intake of 400 I.U. of vitamin

[4] To supply the phosphorus contained in 18% casein for which the salt mixture was designed.

[5] Vitamin-test casein, General Biochemicals, Inc., was used throughout these experiments.

[6] See footnote 5.

A, 4 I.U. of vitamin D, 0.7 mg of α-tocopherol, and 0.06 mg of 2-methyl-1, 4-naphthoquinone.

The composition of the essential amino acid mixture "A" used in the preliminary studies is given in table 1. To provide 5.6% of the physiologically active essential amino acids in

TABLE 1

Composition of the essential amino acid mixtures "A" and "B" [1]

AMINO ACID	PHYSIOLOGICALLY ACTIVE FORM	AS USED [2]	NITROGEN
	%	%	%
L-Lysine monohydrochloride	1.0	1.24	0.190
L-Histidine monohydrochloride monohydrate	0.4	0.54	0.108
L-Arginine monohydrochloride	0.2	0.25	0.066
DL-Tryptophan	0.2	0.20	0.027
DL-Phenylalanine	0.7 (0.9)	0.70 (0.9)	0.059 (0.076)
L-Leucine	0.8	0.80	0.086
DL-Isoleucine	0.5	1.0	0.107
DL-Threonine	0.5	1.0	0.118
DL-Methionine	0.6	0.6	0.056
DL-Valine	0.7	1.4	0.168
Total	5.6 (5.8)	7.73 (7.93)	0.985 (1.002)

[1] The figures in parentheses refer to essential amino acid mixture "B."

[2] Amino acids as supplied by the manufacturers (mostly Merck and Interchemical) were used after checking their purity by microbiological methods (Henderson and Snell, '48). Various samples of commercially available DL-isoleucine showed from 35 to 100% of the expected activity for *Lactobacillus delbrückii 3*, presumably because of contamination with DL-alloisoleucine (Smith and Greene, '48). The samples used in these studies ranged from 76 to 80% pure by this criterion and correspondingly larger amounts were included in the mixtures. It was assumed that the nitrogen of this impurity was available to the rats as non-essential amino acid nitrogen.

the proportions proposed by Rose and co-workers (Rose, '37: Womack and Kade, '44), it was necessary to add 7.73% of the amino acids used, in order to correct for the inactive D-forms of isoleucine, valine and threonine, and the HCl and water of crystallization of the salts of the basic amino acids.

RESULTS

The results of preliminary studies with amino acid-containing rations are presented in table 2. When essential amino acids at a level of 5.6% were the sole soucre of nitrogen, the growth rate was very poor (0.41 gm per day). The addition of 2% glutamic acid improved the growth rate to about 1.3 gm per day; a further increase in glutamic acid to 6% resulted in little improvement. On the other hand, the addition of 2%

TABLE 2

Results of preliminary studies with amino acid rations[1]

NITROGENOUS COMPONENTS	N CONTENT OF RATION	NO. OF ANIMALS	GROWTH RATE	
			Average	Range
	%			gm/day
5.6% EAA	0.98	3	0.41	0.28–0.57
5.6% EAA + 2% GA	1.17	3	1.3	1.2 –1.4
5.6% EAA + 6% GA	1.55	6	1.6	1.4 –1.8
5.6% EAA + 2% GA + 2% casein	1.48	3	2.7	2.6 –2.8
5.6% EAA + 2% GA + 2% AHC	1.48	3	2.3	2.2 –2.3
5.6% EAA + 0.2% DL-phenylalanine + 5.8% GA	1.55	11	2.5	2.1 –2.9

[1] EAA = Essential amino acid mixture "A" shown in table 1.
GA = L-Glutamic acid.
AHC = Acid hydrolyzed casein.

glutamic acid and 2% casein or acid hydrolyzed casein gave growth rates of 2.7 and 2.3 gm per day, respectively. To determine whether this beneficial effect was due to the supply of an essential amino acid in which the mixture was limiting, a number of experiments with smaller numbers of animals were conducted wherein the essential amino acids were added individually and in groups of two and three. The results were negative in all cases (data not presented) except that the addition of 0.2% DL-phenylalanine resulted in an improved rate of growth (2.5 gm per day). At about the same time

Rose and Womack ('46) reported that 0.9% phenylalanine was required with their revised ration. Accordingly the essential amino acid mixture "A" was modified to contain 0.9% phenylalanine and the resulting mixture "B" (table 1) was used in all subsequent experiments reported.

A number of modifications involving the other major ingredients of the ration were made in an effort to improve the growth rates further. Isocaloric replacement of sucrose by 25 gm of corn oil, doubling the vitamin content, the replacement of corn oil and a part of the sucrose by dextrin, lard and agar as used earlier by Rose and Womack ('46), the addition of 5% celluflour or replacement of the sucrose by dextrin, resulted in no improvement. The observation of Borman et al. ('46) on the adverse effect of high levels of fat was confirmed. In several instances during the course of this work 2% lyophilized liver was used and a slight stimulation in growth was observed, probably due to the protein supplied. Antipernicious anemia liver preparations administered intraperitoneally did not stimulate the growth of rats receiving a number of the amino acid rations.

The effect of varying the levels of glutamic acid and the essential amino acid mixture was next studied (table 3). When the glutamic acid level was increased from 5.8% to 15.8% (rations 1 and 3), there was a slight increase in the average rate of growth (2.5 ± 0.07 and 2.7 ± 0.08 gm per day), but this difference was not statistically significant ($t = 1.83$, $P = 0.087$). It is to be noted, however, that comparatively high levels of glutamic acid seem to be well tolerated by the growing rat. Therefore, this amino acid was used in many subsequent experiments for adjusting the nitrogen content of the rations to the desired level. The results with rations 1 and 2 (table 3) suggested a slight depression in growth rate when the essential amino acid mixture was increased from 5.8% to 7.7% at the expense of glutamic acid. Doubling the level of the mixture (compare rations 3 and 4) resulted in a depression of the growth rate from 2.7 ± 0.08 to 2.3 ± 0.11

gm per day, the difference being statistically significant ($t = 2.96$, $P = 0.014$).

Since the rate of growth of rats receiving rations containing amino acids was much less than that of rats receiving equivalent amounts of casein, a number of experiments (see table 4) were conducted to determine whether this poor performance was due to the lack of strepogenin. For this purpose, an acid hydrolysate of casein was prepared as previously described (Henderson et al., '47). The hydrolysate was tested microbiologically (Woolley, '48) and found to be

TABLE 3

Effect of varying levels of the essential amino acid mixture "B" and glutamic acid

RATION NO.	NITROGENOUS COMPONENTS	N CONTENT	NO OF ANIMALS	GROWTH RATE	PROTEIN EFFICIENCY[1]
		%		*gm/day*	
1	5.8% EAA[2] + 5.8% GA	1.55	11	2.5 ± 0.07	3.23
2	7.7% EAA + 2.3% GA	1.55	2	2.3	3.00
3	5.8% EAA + 15.8% GA	2.5	9	2.7 ± 0.08	2.08
4	11.6% EAA + 5.25% GA	2.5	4	2.3 ± 0.11	2.02

[1] Protein efficiency represents the gain in body weight in grams per gram of protein ($N \times 6.25$) consumed.

[2] See footnote, table 2.

free of strepogenin; however, it contained the expected amounts of all essential amino acids other than tryptophan (Henderson and Snell, '48). When a ration containing 20% of this hydrolysate supplemented with 0.2% DL-tryptophan and 0.2% L-cystine was fed to rats, the growth rate averaged 4.25±0.13 gm per day, while the rats fed an equivalent level of casein (19%) grew at the rate of 4.61±0.15 gm per day. The difference between these growth rates was not significant ($t = 1.82$, $P = 0.082$). Replacement of part of the hydrolysate by 5% intact casein or 5% egg albumin purified according to the method of Woolley ('46) gave average growth rates of 4.55±0.14 and 4.40±0.09 gm per day, respectively. From

these results it was concluded that the lack of strepogenin does not limit the growth of young rats significantly.

In view of the fact that the growth rate and protein efficiency vary with the level of protein in the ration, it seemed desirable to maintain a constant level of nitrogen in all rations. In all subsequent experiments, therefore, a nitrogen level of 2.5% was used. This amount is the same as that provided by 19% air dried casein, which is generally considered adequate for optimum growth in the rat. The results of these experiments are presented in table 5.

TABLE 4

Comparison of acid hydrolyzed casein and intact proteins

NITROGENOUS COMPONENTS	NO. OF ANIMALS	GROWTH RATE	PROTEIN EFFICIENCY [1]
		gm/day	
19% Casein	11	4.61 ± 0.15	2.67 ± 0.03
20% AHC + 0.2% DL-tryptophan + 0.2% L-cystine	11	4.25 ± 0.13	2.59 ± 0.06 [2]
14.5% AHC + 0.2% DL-tryptophan + 0.2% L-cystine + 5% casein	11	4.55 ± 0.14	...
14.5% AHC + 0.2% DL-tryptophan + 0.2% L-cystine + 5% egg albumin	3	4.40 ± 0.09	...

[1] See footnote 1, table 3.

[2] Based on 8 of the 11 animals.

While the rate of growth with rations containing 5.8% essential amino acids plus 15.8% glutamic acid was 2.7±0.08 gm per day, the isonitrogenous substitution of 5% casein for glutamic acid increased the growth rate significantly to 3.4±0.13 gm per day. Since earlier experiments had failed to show any improvement with higher levels of the essential amino acids, singly or collectively, it seemed probable that this beneficial effect of casein was due to its non-essential amino acids. This impression was confirmed when a similar rate of growth (3.4±0.12 gm per day) was obtained with the

addition of 6.12% of an arbitrary mixture of non-essential amino acids.[7]

Many experiments of an exploratory character, with two animals in each group, were conducted to ascertain which of these non-essential amino acids was responsible for the observed growth stimulation. By adding the amino acids singly and in various combinations, at the expense of glutamic acid, it was found that glycine, cystine, alanine, serine, proline, and asparagine (or aspartic acid) had negligible effects on the growth rate; figures for these groups are therefore not reported in table 5.

In preliminary experiments a stimulatory effect of L-tyrosine was noted when it was added to rations containing only the essential amino acids and glutamic acid. In the presence of 0.75% arginine hydrochloride, tyrosine increased the growth rate from 2.6 to 3.6 gm per day (rations 4 and 5, table 5). The further addition of 0.5% DL-serine and 1.2% DL-alanine (rations 6 and 7) did not produce any significant change. An attempt was made (rations 13 and 14) to determine whether the growth stimulatory effect of tyrosine could be duplicated by additional amounts of phenylalanine. The basal ration used resembled ration 9 but contained no tyrosine. When 0.5% of either L-tyrosine or DL-phenylalanine was added, the growth rate was approximately the same. Further work, with larger numbers of animals and wider ranges of tyrosine and phenylalanine concentrations, is needed before it can be determined whether the stimulatory action of tyrosine is due to an inadequate phenylalanine intake or to a limited rate of formation of tyrosine from phenylalanine.

In the presenec of 14.3% glutamic acid (rations 1 and 4) additional arginine had no effect on growth. Comparison of rations 9 and 12 indicated that when all but 2% of the glutamic

[7] Five-tenths per cent DL-serine, 0.7% glycine, 1.4% L-tyrosine, 0.2% L-cystine, 0.9% L-proline, 1.22% L-aspargine and 1.2% DL-alanine. This same mixture was used at various levels in the other experiments described here.

TABLE 5

Effect of non-essential amino acids on growth [1]

RATION NO.	NITROGENOUS COMPONENTS [2]	NO. OF ANIMALS	GROWTH RATE	PROTEIN EFFICIENCY [3]
			gm/day	
1.	5.8% EAA + 15.8% GA	9	2.7 ± 0.08	2.08 ± 0.05
2.	5.8% EAA + 5% casein + 8.8% GA	4	3.4 ± 0.13	2.32 ± 0.05
3.	5.8% EAA + 6.2% GA + 6.1% NEAA	4	3.4 ± 0.12	2.29 ± 0.01
4.	5.8% EAA + 14.3% GA + 0.5% L-arg. HCl	3	2.6 (2.4–3.0) [4]	2.1 (1.9–2.3)
5.	5.8% EAA + 13.1% GA + 0.5% L-arg. HCl + 1.4% L-tyrosine	3	3.6 (3.6–3.8)	2.44 (2.29–2.63)
6.	5.8% EAA + 12.4% GA + 0.5% L-arg. HCl + 1.4% L-tyrosine + 0.5% DL-serine	3	3.8 (3.6–4.0)	2.51 (2.41–2.56)
7.	5.8% EAA + 10.4% GA + 0.5% L-arg. HCl + 1.4% L-tyrosine + 0.5% DL-serine + 1.2% DL-alanine	3	3.8 (3.4–4.0)	2.37 (2.37–2.37)
8.	5.8% EAA + 9.2% NEAA + 0.5% L-arg. HCl	5	3.5 ± 0.18	2.48 ± 0.04
9.	5.8% EAA + 7.85% NEAA + 0.5% L-arg. HCl + 2% GA	9	4.1 ± 0.12	2.50 ± 0.05
10.	5.8% EAA + 6.56% NEAA + 0.5% L-arg. HCl + 4% GA	3	3.4 (3.1–3.8)	2.38 (2.26–2.48)
11.	5.8% EAA + 4.9% NEAA + 0.5% L-arg. HCl + 8% GA	3	3.8 ± 0.20	2.39 (2.06–2.64)
12.	5.8% EAA + 8.85% NEAA + 2% GA	3	3.0 (2.9–3.1)	2.37 (2.31–2.48)
13.	5.8% EAA + 6.74% NEAA (no tyrosine) + 0.5% L-arg. HCl + 2% GA + 0.5% L-tyrosine	3	3.8 (3.4–4.0)	2.43 (2.36–2.52)
14.	Same as ration 13, but 0.5% DL-phenylalanine instead of tyrosine	3	3.5 (2.9–4.2)	2.21 (2.0–2.6)

[1] Nitrogen content of all the rations was 2.5%.

[2] EAA = essential amino acid mixture "B" shown in table 1. NEAA = non-essential amino acid mixture (see text). See also footnote, table 2.

[3] See footnote 1, table 3.

[4] Range.

acid was replaced by the non-essential amino acid mixture additional arginine was beneficial.

In another series of experiments (rations 8 to 11) the effect of varying the concentration of glutamic acid was studied, the level of the non-essential amino acid mixture being altered to adjust the nitrogen content to 2.5% in each case. Comparison of rations 8 and 9 suggested a stimulation of growth by 2% glutamic acid. Higher levels (rations 9–11) did not produce quite as good growth, possibly because of a simultaneous reduction in the other non-essential amino acids. This question was examined more critically by Rose, Oesterling and Womack ('48). They found that the removal of glutamic acid from a diet containing 19 amino acids resulted in a slight inhibition of growth which was of doubtful statistical significance.

It is to be noted that ration 9 gave the best growth of all the amino acid-containing rations studied. The average growth rate was 4.1 ± 0.12 gm per day and the protein efficiency averaged 2.5 ± 0.05 gm per gram of protein consumed. The corresponding figures for a 19% casein ration were 4.61 ± 0.15 gm per day and 2.67 ± 0.03 gm per gram of protein. This improvement on ration 9 seemed to be the result of the growth-stimulating effects observed earlier with 0.5% arginine hydrochloride, 2% L-glutamic acid and 1.8% L-tyrosine (supplied by the non-essential amino acid mixture).

In all of these experiments it was observed that the rats receiving the free amino acids grew at reduced rates during the first week compared with the rats receiving casein. This was presumably due to the poor palatability of the amino acids as evidenced by the lower food consumption. As they became accustomed to the rations, however, the animals ate well and the growth rate during the third and 4th weeks was improved. However, the rats appeared incapable of completely overcoming the effects of the initial handicap. To obviate this difficulty forced feeding was used, and thereby the food intake of the amino acid-fed rats was equalized with that of the casein-fed rats. The ration was homogenized in a

TABLE 6

Effect of forced, paired-feeding of amino acid and casein rations[1]

EXP. NO.	GROUP NO.	NITROGENOUS COMPONENTS	METHOD OF FEEDING	NO. OF ANIMALS	GROWTH RATE	PROTEIN EFFICIENCY[2]
					gm/day	
I	1	19% casein	Ad libitum	3	4.8 (4.8–5.6)[3]	2.78 (2.67–2.92)
	2	19% casein	Forced, paired with group 1	4	4.8 (4.0–5.4)	2.77 (2.34–3.13)
	3	5.8% EAA + 4.9% NEAA + 8% GA + 0.5% L-arg. HCl	Ad libitum	3	3.6 (3.3–3.8)	2.39 (2.06–2.64)
	4	5.8% EAA + 4.9% NEAA + 8% GA + 0.5% L-arg. HCl	Forced, paired with group 1	4	4.4 (4.3–4.6)	2.46 (2.39–2.51)
II	5	19% casein	Ad libitum	3	4.25 (3.9–4.4)	2.63 (2.'6 -2.72)
	6	5.8% EAA + 7.85% NEAA + 2% GA + 0.5% L-arg. HCl	Ad libitum	3	4.1 (3.7–4.5)	2.53 (2.42–2.69)
	7	5.8% EAA + 7.85% NEAA + 2% GA + 0.5% L-arg. HCl	Forced, paired with group 5	3	4.3 (4.2–4.5)	2.57 (2.48–2.63)

[1] EAA = essential amino acid mixture "B." See table 1, as well as footnote, table 2. NEAA = non-essential amino acid mixture. See text.

[2] See footnote 1, table 3.

[3] Range.

small volume of water using a Potter-Elvehjem homogenizer to give a thick slurry. It was administered by stomach tube in three feedings at 5-hour intervals daily. The results of two such paired feeding experiments (see table 6) showed that the increased intake of the amino acid rations resulted in a better growth rate, approaching that of rats receiving casein rations ad libitum. Forced feeding per se did not have any adverse effect, as can be seen from the data relating to groups 1 and 2. Comparison of groups 3 and 4 and groups 6 and 7 showed that the forced feeding of the amino acids did not reduce the protein efficiency values. It should be noted that these values closely paralleled the growth rates at a given nitrogen level. For example, the correlation coefficient, r, for protein efficiency values and growth rates in table 5 is 0.67. From these observations made on more than 200 rats it appears that food consumption figures are of no special value in studies of this type.

DISCUSSION

The results obtained in the course of this investigation confirm the observations made by Rose and associates regarding the inadequacy of 0.7% phenylalanine (Rose and Womack, '46) and the improved growth resulting from the addition of the non-essential amino acids to adequate amounts of the essential amino acids (Rose et al., '48). It is not possible to make a quantitative comparison of the present results and those obtained by the above workers. The chief points of difference are with regard to arginine and tyrosine. Borman et al., ('46) stated in a footnote that 0.25% arginine hydrochloride was adequate for growth and that larger percentages were not more effective under the conditions of their tests. In the present series of experiments, additional arginine was found beneficial. Similarly, Womack and Rose ('46b) observed that the addition of tyrosine failed to accelerate the growth rate when the diet contained an adequate amount (0.9%) of phenylalanine. The studies reported here indicate a growth stimulation with tyrosine.

A number of differences in the experimental rations and the way in which the experiments were designed may account for these apparent discrepancies. In the experiments described here the essential amino acids were used at the levels tentatively suggested as minimum (Rose, '37; Womack and Kade, '44), contributing only 5.8% of physiologically available forms. Rose and Womack (Rose and Womack, '46; Womack and Rose, '47) have used the essential amino acids at levels of 25 to 100% above these minimum requirements, giving approximately 8.6% of the active forms. As a result of this and the rather low levels of non-essential amino acids fed, the ration of non-essential amino acid nitrogen to essential amino acid nitrogen in the mixtures used by these workers was somewhat lower than those in the rations we used. Finally, in all except their recent experiments Rose and co-workers (Borman et al., '46; Womack and Rose, '47; Rose et al., '48) employed rations containing 31% fat.

The non-essentiality of tyrosine was established by experiments in which limited growth was obtained with no dietary source of this amino acid. Abundant evidence has been presented of its formation from phenylalanine in the rat. Whether the conversion of phenylalanine to tyrosine is rapid enough to permit optimum rates of growth is not known. This is a possible explanation for the stimulatory action of tyrosine in these experiments. Womack and Rose ('46b) established the phenylalanine requirement and the extent to which tyrosine will substitute for phenylalanine in experiments where the growth rate did not exceed 2 gm per day in most cases. It seems possible that (a) the phenylalanine requirement may be more that 0.9% for optimum growth without dietary tyrosine or (b) the rate of formation of tyrosine may become limiting as the growth rate increases. Further, the capacity for this conversion may be affected by the nature of the ration. Such a concept is in keeping with the recent findings of Rose and co-workers ('48) regarding the need for glutamic acid. The results reported here could be explained by either of the above alternatives, but it is not

possible to distinguish between them from the data thus far obtained. It should be noted that in no case did the growth exceed 3.0 gm per day without tyrosine or additional phenylalanine, while growth rates of 3.6 gm per day were obtained with 12 amino acids. Similar considerations may explain the failure of Borman et al., ('46) to obtain better growth with 0.6% arginine than with 0.2%. As has been pointed out (Womack and Rose, '47), the stimulatory effect of arginine was very small until improvements were made in the rations. It seems possible that still other of the so-called non-essential amino acids may be shown to be stimulatory as the growth rates approach more closely those observed with good quality protein.

Although the improvements made in the amino acid mixtures resulted in growth rates as high as 4.1 and 4.4 gm per day under conditions of ad libitum and forced feeding, respectively, it should be noted that the growth performances were still slightly inferior to those of rats receiving casein. Computations from the data on the 11 animals receiving the casein diet (table 4) and the 9 rats fed the best amino acid ration (group 9 in table 5) show that the difference between these groups is significant, the P value being 0.03. Further work is needed to find an amino acid mixture that will support a growth rate as good or better than casein. The inferiority of the present amino acid rations may possibly be due to the presence of the unnatural D-isomers. In the formulation of these rations it has been assumed that the D-amino acids serve to supply non-essential amino acid nitrogen. At the levels used they may not produce any symptoms of toxicity but may cause a slight depression of growth. On a ration containing a mixture of synthetic (racemic) amino acids, Wretlind ('48) obtained a growth rate of 0.33 gm/day at a 10% level and 0.89 gm/day at 20%, while at 30 and 40% levels the animals lost weight. He concluded that synthetic amino acids contain some toxic material. In these laboratories an alkaline hydrolysate of casein was prepared by autoclaving with barium hydroxide. Microbiological assays (Henderson

and Snell, '48) for the essential amino acids in the hydrolysate showed that arginine and threonine were completely destroyed and several others, particularly valine, histidine and isoleucine, suffered partial destruction. This hydrolysate was fed to rats at a 20% level, supplemented with the essential amino acids to correct for these losses. The animals ate very little of the ration and died within a week. This was probably not due to D-amino acids alone but to the presence of toxic products formed during the hydrolysis. There is no report of any rat growth studies employing only the natural forms of the amino acids. However, Brand and Bosshardt ('48) have reported that mice fed a mixture of L-amino acids duplicating the composition of β-lactoglobulin grew at the same rate as those receiving a corresponding amount of the native protein. When certain DL-amino acids were used in the mixture, the growth response was impaired.

In these laboratories, Maddy and Elvehjem ('49) have fed a balanced mixture of 16 amino acids to mice and obtained a rate of growth closely approaching that obtained with casein. Their work also emphasizes the importance for optimum growth of adding some of the non-essential amino acids. Possibly an imbalance of the amino acids in the mixtures employed in these studies could account for their inferiority as a source of nitrogen for the growing rat. Grau ('48) has presented evidence that the lysine requirement for the growth of the chick is a direct function of the protein content of the ration. Work in this laboratory (Hankes et al., '48) on the niacin-tryptophan relationship has shown that slight increases in the concentrations of certain amino acids cause marked reduction in the growth rate of rats, again suggesting the importance of a proper balance of amino acids in rations where the levels of certain dietary essentials are marginal.

SUMMARY

A study was made of the growth of weanling rats receiving rations containing mixtures of purified amino acids, acid

hydrolyzed casein and intact casein. Daily food consumption was recorded and data on the protein efficiency of the different rations are presented.

The growth rate of young rats fed 20% acid hydrolyzed casein supplemented with 0.2% DL-tryptophan and 0.2% L-cystine was not significantly less than that of rats receiving an equivalent amount of intact casein, offering no support for the presumption that the rat requires a source of strepogenin under these conditions.

Improved rations containing 18 amino acids to provide a nitrogen level of 2.5% gave average growth rates of 4.1 and 4.4 gm per day under conditions of ad libitum and forced, paired feeding, respectively. These values represent 80 to 90% of the growth rate of rats receiving ad libitum a ration containing casein at an isonitrogenous level. Evidence presented indicates that the improved growth rate was the result of increasing the level of arginine hydrochloride to 0.75% and adding 2% L-glutamic acid and 1.8% L-tyrosine to a ration containing other "non-essential" amino acids and the minimum levels of the 10 essential amino acids.

The probable reasons for the slight inferiority of the amino acid-containing rations to the casein-containing ration are discussed.

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THE CHOLINE REQUIREMENT OF THE BABY PIG

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THIRTY FIGURES

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In a previous paper it was established that the baby pig on a 30% casein ration requires dietary choline, and the deficiency syndrome was described (Johnson and James, '48a). The object of the experiments reported in this paper was to determine the dietary choline level esssential for optimum growth and for the physiological well-being of the pig.

To determine the choline requirement it is necessary to consider the methionine (or other methyl donor) content of the diet. A choline deficiency was produced in the baby pig using a diet containing approximately 0.8% methionine, based on microbiological assays of casein (Henderson and Snell, '48; Horn et al., '46; Stokes et al., '45) to 1.0% based on chemical assays (Block and Bolling, '45). This suggests that in the baby pig the methylation of aminoethanol by the methyl groups from methionine does not take place to any considerable extent (compare with the chick as discussed by Jukes, '41).

EXPERIMENTAL

Two-day-old Duroc-Jersey pigs from the University farm were used in this study. The technique of feeding and care of the animals has been described (Johnson, James and Krider, '48b). The composition of the basal ration is given

in table 1. As in previous experiments, 2% sulfasuxidine [1] was included in the ration to inhibit intestinal synthesis. Two experiments were conducted; one using ad libitum feeding to establish the range of the requirement and the second using

TABLE 1

Composition of the basal diet

	%
Casein (Labco, vitamin-free)	30.0
Glucose (cerelose)	37.4
Mineral salts [1]	6.0
Lard	26.6

These materials were made up into a "milk" containing 4% lard (liquid basis) and the following amounts of vitamins [2] per liter:

Vitamin A	2000 I.U.	Pyridoxine	2.67 mg
Vitamin D_3	200 I.U.	Ca-pantothenate	8.00 mg
Alpha-tocopherol acetate	1.0 mg	Inositol	26.00 mg
2-methyl-1,4-naphthoquinone	0.26 mg	PABA	2.67 mg
Thiamine	0.65 mg	PGA	0.052 mg
Riboflavin	1.33 mg	Biotin	0.01 mg
Nicotinic acid	2.67 mg		

Choline chloride was added as indicated in the text. Reticulogen [3] was added at the rate of 0.25 ml/day in test 2.

[1] See Johnson et al., '48b.

[2] Thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, biotin, nicotinic acid and α-tocopherol acetate were supplied by Hoffmann-La Roche, Inc., Nutley, New Jersey, through the courtesy of Dr. J. C. Bauernfeind. Pteroylglutamic acid was supplied by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, New York, through the courtesy of Dr. T. H. Jukes. Inositol was supplied by the A. E. Staley Manufacturing Co., Decatur, Illinois. Hyflavin (a highly water-soluble form of riboflavin) was supplied by Endo Products, Inc., Richmond Hill, New York.

[3] Reticulogen was supplied by Eli Lilly and Co., Indianapolis, Ind.

paired feeding to eliminate any effects due to differences in food intake within the limits of this range.

In the first experiment 4 groups of three pigs each were fed ad libitum the basal ration plus choline chloride at the

[1] Sulfasuxidine was supplied by Sharp and Dohme, Inc., Glenolden, Pa., through the courtesy of Dr. S. F. Scheidy.

following levels based on the dry matter of the ration: Group I — 0.0%, II — 0.05%, III — 0.10% and IV — 0.20%. The experiment was continued for 8 weeks. At its conclusion representative pigs from each group were sacrificed and microscopic sections were made of their livers, kidneys, nerves, and skeletal muscles.

From the results of experiment 1 the dietary requirement for choline chloride appeared to be between 0.05% and 0.1%. Therefore, in the second experiment these two levels of choline were compared by pair feeding 6 pairs of pigs for 8 weeks, as in experiment 1. Reticulogen, a liver extract material, was added to the basal ration in this experiment at the rate of 0.25 ml daily to supply an essential growth factor or factors, as it had been found by the present authors in previous work that the basal diet was deficient in this respect (Neumann et al., '48). All 12 of the pigs in experiment 2 were sacrificed and microscopic sections made of their tissues as in experiment 1. In addition, ether extract determinations were made of the livers.

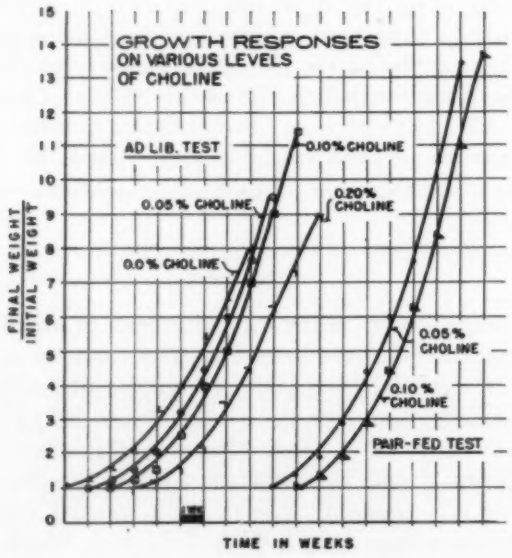
RESULTS

The average growth curves of the groups of pigs from both experiments are plotted in chart 1. The rates of gain of the 4 groups in experiment 1 are not significantly different, although the difference between groups I and III is indicative of a growth effect ($P = 0.08$). However, this effect as shown in experiment 2 and as reported previously (Johnson and James, '48a) is probably due to an increased food intake. Similar findings have been reported by Foá, Weinstein, and Kleppel ('48) for the rat. These workers found no effect of a choline deficiency on the growth of the rat when food consumption was equalized. From the data in table 2 it is apparent that the 0.05% and 0.10% choline groups made increasingly more rapid gains on less dry matter consumed than the negative control group, although the "P" value between the gains of the 0.0% and 0.1% choline groups is only 0.08%.

Pictures of two representative pigs from each of the first three groups in experiment 1 are given in plate 1. Pigs in figures 1 and 2 are from the "0.0% choline" group; those in 3 and 4 from the "0.05% choline" group; and the animals in 5 and 6 from the "0.1% choline" group. The pigs in figures 1 to 4, and in particular 2 and 4, show the gross

CHART 1

Graph showing the average growth responses on the various levels of choline. Note similarity of the growth pattern, regardless of level fed or method of feeding used, i.e., freedom from a plateau in the growth curve even on the choline-free diet.



symptoms of choline deficiency. They are unthrifty and have poor conformation, appearing short-legged and pot-bellied. These pigs lacked coordination in their movements (as also reported by Ellis, Madsen and Miller, '43) and seemed to lack proper rigidity in the joints, particularly in the shoulders (note also pigs 8 and 9, plate 1, Johnson and James, '48a).

The microscopic sections of nerve and skeletal muscle failed to show any differences between groups.

The performance records of the baby pigs in experiment 2 are given in table 3. The two groups showed no difference in rate or economy of gain.

Three pairs of pigs from experiment 2 are pictured in plate 2. Pigs in figures 7, 9, and 11 received 0.05% choline, while those in figures 8, 10 and 12 received 0.1%. The pigs on the higher level of choline were thriftier in appearance and had better haircoats than their pair mates on the lower level.

The histological findings with respect to the livers and kidneys of pigs from both experiments are illustrated in

TABLE 2

Response of baby pigs fed various levels of choline ad libitum for 56 days

DIETS FED	BASAL + 0.00% CHOLINE	BASAL + 0.05% CHOLINE	BASAL + 0.10% CHOLINE	BASAL + 0.20% CHOLINE
No. of pigs fed	3	3	3	3
Ave. initial weight (kg)	1.67	1.60	1.55	1.70
Ave. final weight (kg)	13.49	15.27	17.56	15.36
Ave. $\frac{\text{final weight}}{\text{initial weight}}$ (kg)	8.07	9.53	11.69	9.22
Dry matter consumed (kg)/kg gain	1.22	1.11	1.07	1.18

plates 3, 4 and 5. In plate 3, figures 13, 14 and 15 are photomicrographs of sections of the livers of pigs in experiment 1 receiving 0.0, 0.5 and 0.10% choline, respectively. Figures 16 and 17 are liver sections from pair-fed pigs in experiment 2 on 0.05 and 0.10% choline, respectively. Note that on the choline-free diet (fig. 13) the hepatic cells of the liver have become greatly distended with fat and the nuclei are pushed over to one side. The livers of the animals on the 0.05% level of choline contain slightly more fat globules than those from the pigs on the 0.10% level. In addition, there was a slight but significant difference in ether extract content of the livers.

Renal damage appeared more serious than the liver condition. The photomicrographs (plates 4 and 5) illustrate the degree of damage to the glomeruli and the tubular epithelium. On the 0.0% level all glomeruli shown are occluded and apparently non-functional (fig. 18), as contrasted with the glomeruli seen in figure 20 from a pig on the 0.1% choline level and in figure 21 from a pig on an adequate farm ration.

The photomicrographs were made of the cortical region of the kidney in all cases, as damage in this area was more pronounced. The kidneys of the pigs on the 0.05% choline level show less damage than those on the 0.0% level but are

TABLE 3

Effect of 0.05 and 0.10% choline in baby pig diets (equal feed intake for 56 days)

DIETS FED	BASAL DIET + 0.05% CHOLINE	BASAL DIET + 0.10% CHOLINE
No. of pigs fed	6	6
Ave. initial weight (kg)	1.60	1.59
Ave. final weight (kg)	21.32	21.79
Ave. $\frac{\text{final wt.}}{\text{initial wt.}}$ (kg)	13.32	13.70
Ave. liver weight (dry) (gm)	146.72	137.17
Ave. ether extract in liver (%)	5.80	5.20
Ave. liver ether extract/kg body wt.	1.34	1.16
Dry matter consumed (kg)/kg gain	1.167	1.140

definitely abnormal. This is true in both the ad libitum feeding experiment (fig. 19) and in the paired feeding experiment (fig. 22), while the kidneys of the pigs on the 0.10% level are normal (figs. 20 and 23). The photomicrographs in plate 5 illustrate at higher magnification the kidneys on both the ad libitum and the paired feeding experiments when less than 0.10% choline was included in the diet.

DISCUSSION

An analysis of the data presented in this paper shows that the very young pig requires choline even in the presence of a substantial amount of methionine. In this respect the inter-

relationship of choline and methionine in the baby pig is similar to that in the chick rather than that in the rat. Jukes ('40) showed that methionine will not prevent the symptom of perosis seen in choline deficiency in the chick; that is, it will not methylate aminoethanol (Jukes, '41) to form choline as it does in the rat (Du Vigneaud, Chandler, Cohn and Brown, '40). Recently McKittrick ('47) has reported that for optimum growth of 4-week-old chicks 0.5% methionine is required when optimum choline (0.55%) is included in the diet, and that 0.10% choline is required when optimum methionine (0.75%) is included in the diet. The baby pigs in our experiments received approximately the same amount of methionine (0.8%) as that which allowed optimum growth with 0.1% choline in these chick experiments. Similarly the pigs showed a definite choline requirement of the same magnitude (0.1%) as that which McKittrick reported for the chick.

However, Treadwell ('48) has recently reported that in the young (50 gm) rat the requirement for methionine on a choline-free diet is 1.3 to 1.8%. While there is no choline requirement at this level of methionine, when the diet contains only 0.8% methionine the young rat has a definite need for choline. With optimum choline the methionine requirement of the rat is 0.5–0.6% (Womack and Rose, '41).

From these data it woud seem necessary to undertake further experiments involving still higher methionine levels to determine whether the baby pig can synthesize all of its required choline in the presence of adequate methionine or whether there is an absolute dietary requirement for choline.

SUMMARY

1. On a "synthetic milk" ration containing approximately 0.8% methionine (30% casein) the baby pig requires 0.1% choline in its diet.

2. It appears that in the young pig, as in the chick, the ability of methionine to supply methyl groups for the synthesis of choline from aminoethanol may be limited. The

methionine requirement of the baby pig must be estabished before this can be determined.

3. When the food intake is equalized choline does not have a growth stimulating effect.

4. Choline deficiency in these baby pigs resulted in gross symptoms of unthriftiness, poor conformation (short-legged and pot-bellied), lack of coordination in movements and a characteristic lack of proper rigidity in the joints, particularly the shoulders. The pigs also showed typical fatty infiltration of the livers and characteristic renal glomerular occlusion and some tubular epithelial necrosis.

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PLATE 1

EXPLANATION OF FIGURES

Figures 1 and 2 represent pigs on the 0.0% choline diet, whereas the pigs represented by figures 3 and 4 were fed on 0.05% choline, and by figures 5 and 6 on 0.10% choline. These pigs were in the ad libitum feeding test.

PLATE 1

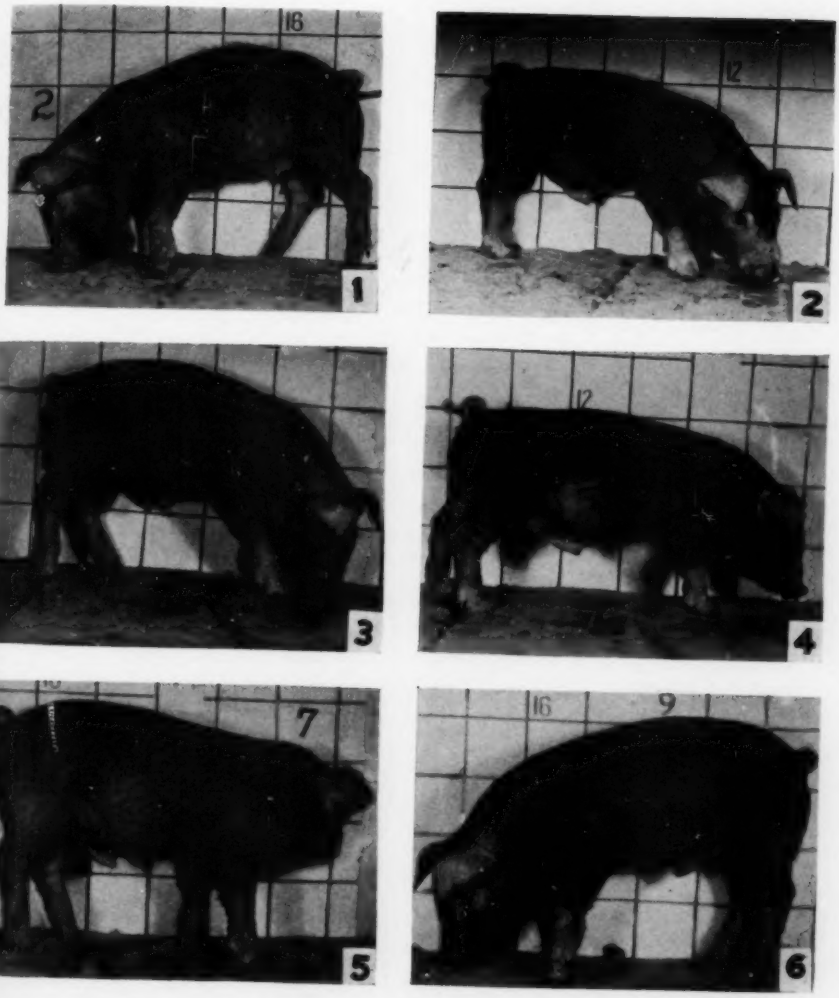
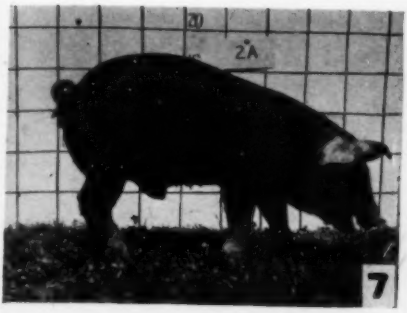


PLATE 2

EXPLANATION OF FIGURES

The figures on the left of the plate, viz., 7, 9, and 11, are photographs of pigs on the 0.05% choline level, whereas those on the right are their pair mates which were fed 0.10% choline. These pigs are typical of the pair-fed test.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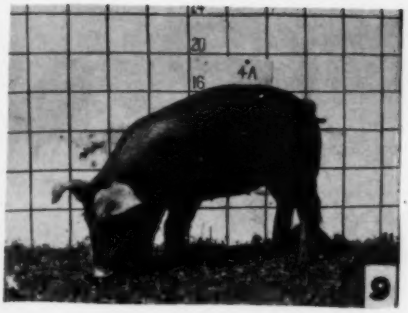
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5A
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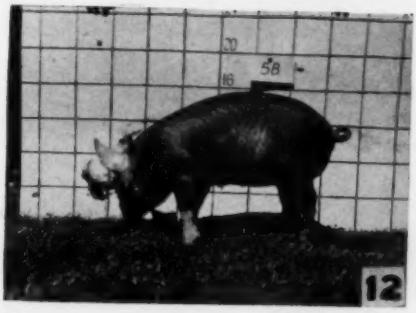
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PLATE 3

EXPLANATION OF FIGURES

Photomicrographs of liver sections of pigs at termination of experiment (8 weeks). Magnification × 225. All sections were made by paraffin method and stained with Harris's hematoxylin and Orange G.

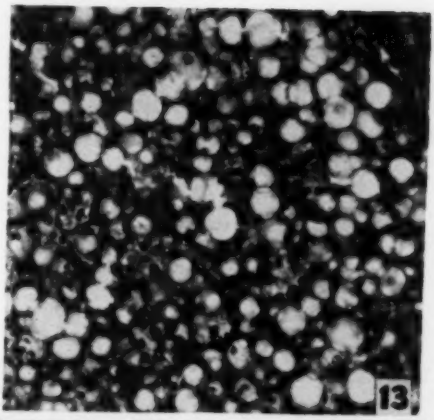
13 Liver from pig on 0.0% level of choline (see fig. 2, plate 1).

14 Liver from pig on 0.05% level of choline (see figs. 3 and 4, plate 1). Note small amount of fatty infiltration.

15 Liver from pig on 0.10% level of choline (see figs. 5 and 6, plate 1). Note absence of abnormal fatty infiltration.

16 Liver from pig on 0.05% level of choline from paired feeding test (see figs. 7, 9, and 11, plate 2). Note small amount of fatty infiltration.

17 Liver from pig on 0.10% level of choline from paired feeding test (see figs. 8, 10 and 12, plate 2).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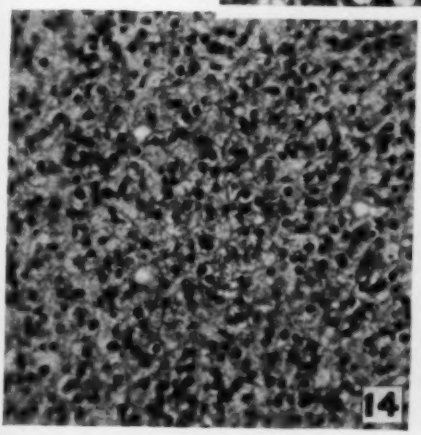
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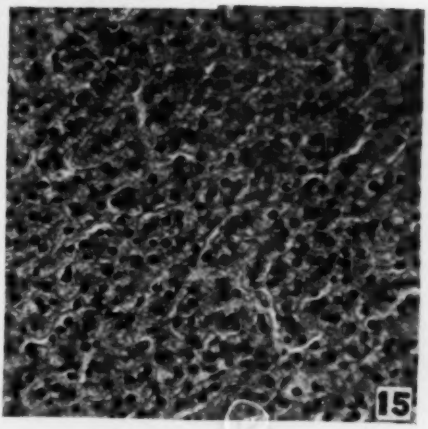
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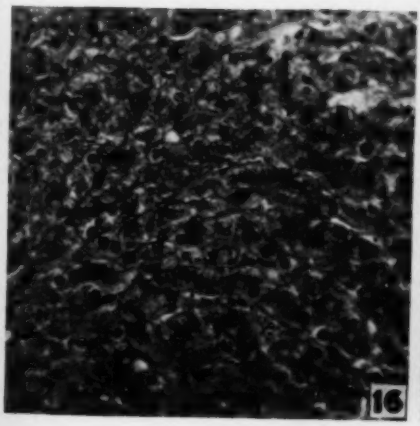
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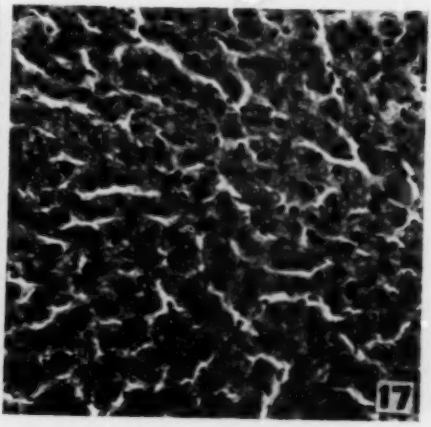
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PLATE 4

EXPLANATION OF FIGURES

Photomicrographs of kidney sections of pigs at termination of experiment (8 weeks). Magnification × 75. All sections were made by paraffin method and stained with Harris's hematoxylin and Orange G.

18 Kidney from pig on 0.0% level of choline. Note extensive pathology of glomeruli and tubules.

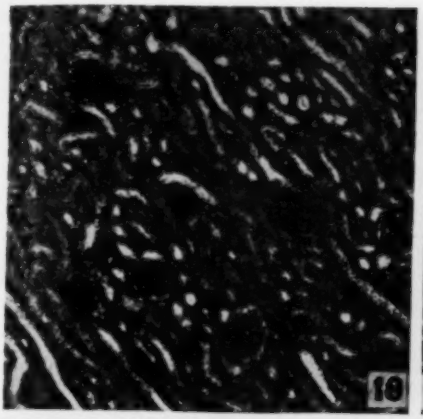
19 Kidney from pig on 0.05% level of choline. Note some normal and some pathological glomeruli and tubules.

20 Kidney from pig on 0.10% level of choline. Note absence of pathological condition.

21 Kidney from pig of same age fed on an adequate farm ration. Note similarity to figure 20.

22 Kidney from pig on 0.05% level of choline from paired feeding test. Note same condition as found in figure 18.

23 Kidney from pig on 0.10% level of choline from paired feeding test Note normal condition and similarity to figures 20 and 21.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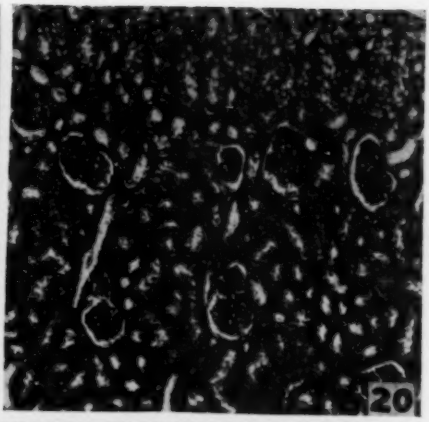
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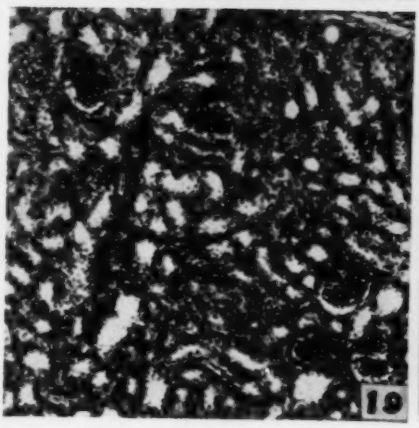
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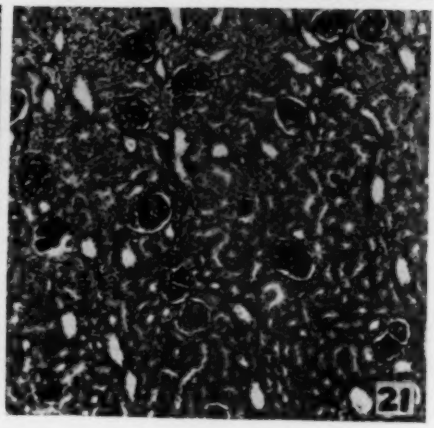
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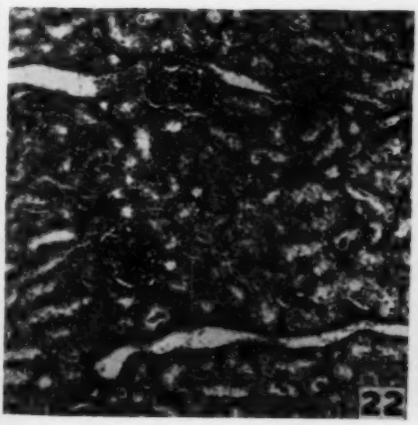
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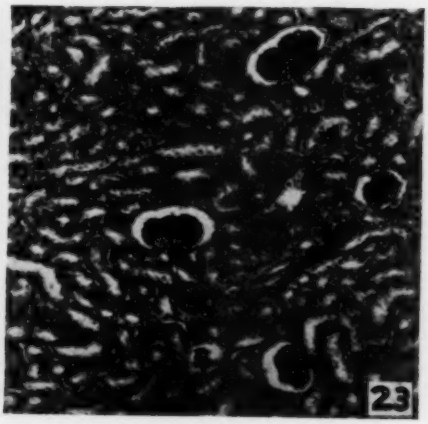
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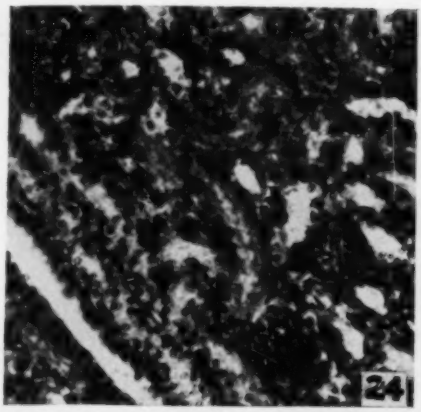
PLATE 5

EXPLANATION OF FIGURES

Photomicrographs of same kidney sections as shown in plate 4. Higher magnification × 225 has been used to show greater glomerular and tubular detail.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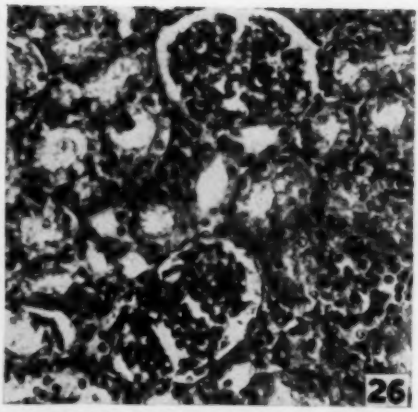
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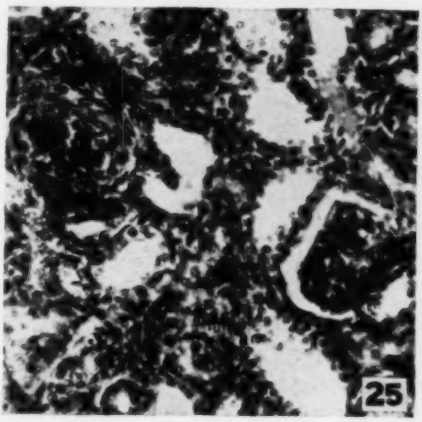
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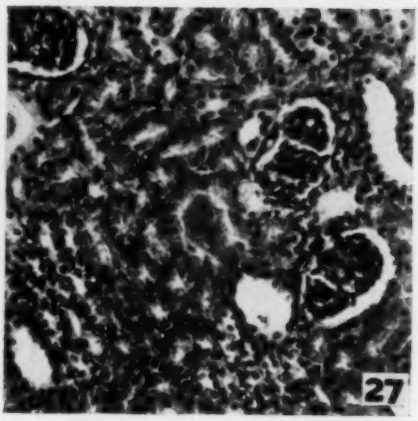
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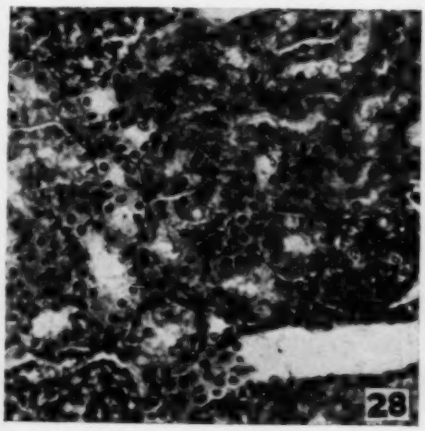
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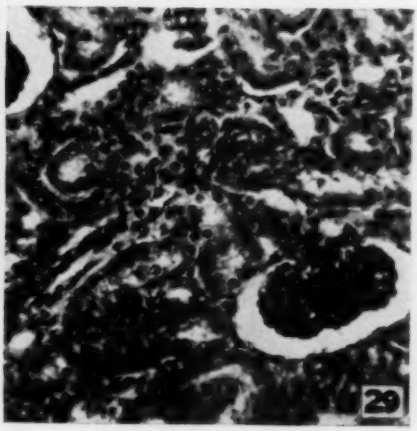
29

THE BIOLOGICAL VALUE OF CORN AND WHEAT PROTEINS IN THE MALE INFANT, WITH A NOTE ON THE UTILIZATION OF D-TRYPTOPHAN [1]

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During the past two years we have made measurements of the food value of some vegetable proteins in the infant. We felt this effort worthwhile for two reasons: (a) vegetable protein diets might prove useful in feeding infants allergic to animal protein diets; and (b) extensive use of vegetable protein diets may soon be necessary if the gloomy predictions of the agronomists regarding the failure of future food production to keep pace with population growth are ultimately realized. Of course, the use of vegetable protein milks (soybean, millet and cornmeal) for infant feeding has long been practiced empirically in many lands.

In this investigation, as in the previous animal tissue study from this laboratory (Albanese and assoicates, '48), we chose to use isolated protein fractions of wheat and corn supplemented with appropiate amounts of lysine and tryptophan rather than the whole products, to avoid gross contamination of the diet by unknown constitutents or chemical structures. The nutritive characteristics of these diets are compared with

[1] The work described in this report was supported by grants from the Biochemical Division of the Interchemical Corporation and the Office of Naval Research.

those of diets in which the protein component was supplied by whole casein, or a tryptophan- and cystine-reinforced acid hydrolysate of casein, or evaporated cow's milk.

Quite apart from the main purpose of this report, some pertinent observations are also related on the value of DL-tryptophan as a substitute for L-tryptophan in reinforcing tryptophan-deficient diets.

TABLE 1

Composition of casein and zein diets
(Daily ration per kilogram of body weight)

DIETS	CASEIN	ZEIN [1]	CTH-2
	gm	*gm*	*gm*
Protein	3.5	3.2	0
Acid hydrolyzed casein	0	0	3.4 [2]
L-Lysine (Interchemical)	0	0.2	0
DL-Tryptophan (Merck)	0	0.1	0.1
L-Cystine (Merck)	0	0	0.4
Crisco	4.0	4.0	4.0
Dextrimaltose (special) [3]	12.0	12.0	12.0
Vitamin B mixture [4]	0.079	0.079	0.079
Salt mixture [5]	1.6	1.6	1.6

[1] Kindly supplied to us by the Corn Products Refining Company as Mazein.

[2] Protein = N × 6.25.

[3] A vitamin-free preparation kindly supplied us by Dr. Warren M. Cox, Jr., of Mead Johnson and Company.

[4] This mixture had the following composition, which was calculated to supply daily the same quantities as a quart of fresh cow's milk (measured in mg): thiamine, 0.4; nicotinic acid, 0.9; pantothenic acid, 3.5; pyrodoxine, 0.7; inositol, 180; p-aminobenzoic acid, 0.5; choline, 147; riboflavin, 170.

[5] The salt mixture employed had the following composition (measured in gm): $FeSO_4$ 0.9, NaCl 6, calcium gluconate 48, $Ca(OH)_2$ 12, KH_2PO_4 7, KCl 6, MgO 0.1.

EXPERIMENTAL

Diets and procedures

The composition of the diets employed in the first and second experiments is shown in tables 1 and 2, respectively. The level of dietary protein, i.e., 3.2 to 3.5 gm/kg of body weight, was determined by an evaluation of the available data in the

literature (Albanese, '47) and our own experience as an intake required for optimum N retention. As in previous studies, these were given in 5 daily feedings and were supplemented daily with 50 mg of ascorbic acid and 15 drops of oleum percomorphum. The composition and preparation of the diets shown in table 1 differed in several respects from

TABLE 2

Composition of corn and wheat gluten diets
(All diets fed at the rate of 100 Cal. and 3.5 gm of protein per kilogram of body weight)

INGREDIENT	DIET	
	Corn gluten	Wheat gluten
	%	%
Wheat gluten [1]	0.00	3.34
Corn gluten [2]	3.25	0.00
L-Tryptophan	0.04	0.00
L-Lysine	0.21	0.16
Brewers' yeast	1.00	1.00
Olive oil	4.00	4.00
Dextrimaltose no. 2	9.60	9.60
Arrowroot starch	2.30	2.30
Salt mixture [3]	1.60	1.60
Water	78.00	78.00
Estimated lysine content (mg/100 gm)	240.00	240.00

[1] Kindly supplied to us by the Biochemical Division of the Interchemical Corporation as Product G.

[2] Kindly supplied to us by the Corn Products Refining Company as Product no. 3323.

[3] The salt mixture employed had the following composition (measured in gm): $FeSO_4$ 0.9, NaCl 6, calcium gluconate 48, $Ca(OH)_2$ 12, KH_2PO_4 7, KCl 6, MgO 0.1.

those which we customarily employ for these bioassays. Instead of brewers' yeast a synthetic mixture of the commercially available B vitamins was used and the diet components were mixed with water to a cereal-like consistency and spoon-fed, in contrast to the milk-like consistency of our usual diets (table 2), which are bottle-fed.

The diet periods were of 7 days' duration and consecutive, but collections of excreta were omitted on week-ends. The

subjects were partially immobilized by specially designed abdominal supports which also held the urinary adapters in place. Daily 24-hour urine specimens were collected in bottles containing 10 ml of 15% (by volume) HCl and 1 ml of 10% alcoholic thymol. The feces were collected in 19 cm porcelain evaporating dishes which were held in place by a properly shaped excavation in the mattress, and the daily stools were

TABLE 3

Comparison of biological value of zein, casein and acid hydrolyzed casein formulae in the normal male infant
(All results given as daily averages of 7-day periods)

SUBJECT	DIET	TOTAL N INPUT	DAILY BODY WEIGHT CHANGE	NITROGEN RETENTION	TOTAL PLASMA PROTEIN	DAILY FECAL N
		gm	*gm*	*mg/kg*	*gm %*	*gm*
	Casein	3.54	+ 12	174	6.90	0.14
	Zein	3.54	— 7	45	7.65	1.97
S.A.	Zein	3.54	+ 5	— 19	5.70	2.21
7 months	Casein	3.54	+ 12	194	5.99	0.17
6,230 gm	Casein	3.72	+ 12	173	5.92	0.21
	CTH-2	4.12	— 8	144	6.41	0.21
	CTH-2	4.12	— 28	137	6.90	0.16
	Casein	3.90	+ 24	178	7.38	0.15
	Zein	3.90	+ 23	80	7.81	1.45
G.O.	Zein	3.90	— 17	56	6.13	1.35
6 months	Casein	3.90	+ 23	132	5.53	0.33
6,965 gm	Casein	3.90	+ 17	136	4.25	0.20
	CTH-2	4.25	+ 17	58	7.12	0.27
	CTH-2	4.25	+ 10	64	7.16	0.23

accumulated under refrigeration for each period in wide-mouth jars containing 200 ml of 70% alcohol. The subjects were weighed daily during the course of the experiment.

The data on nitrogen retention were calculated from the results of nitrogen determinations on the 24-hour urine collections, analyses of the pooled feces from each period, and computations of the daily N intake based on food consumption records and the determined N content of the diets.

Blood samples were collected over lithium oxalate by vena puncture on the last day of each diet period. The hemoglobin concentration of these specimens was determined colorimetrically in the Klett-Summerson photoelectric colorimeter. The total plasma proteins, albumin, globulin and non-protein N were determined by the procedure described by some of the present authors (Albanese, Irby and Saur, '46).

RESULTS AND DISCUSSION

First experiment

The most striking feature of this study is the finding with respect to the very poor biological value of the supplemented zein diet (table 3). The nutritional inadequacy of zein for the rat has been known since the work of Osborne and Mendel (Mendel, '15), but they found that the deficiencies could be corrected by the addition of tryptophan and lysine to the diet. The increase in fecal N output (some 50% or more of the intake) observed during the zein diet period indicates that this apparent species difference can be ascribed primarily to poor assimilation of zein by the infant. Isolation experiments disclosed that the extra fecal N occurring in the stools collected during this diet period was in the form of whole zein, which could be readily isolated from the stools. *In vitro* experiments disclosed that in 6 hours commercial digestive enzymes, pancreatin and trypsin derived from hog tissues, hydrolyzed zein to about one-third the amino N level attained with casein under comparable conditions. From these evidences it appears that the poor nutritional quality of zein in the infant is to be attributed primarily to its poor digestibility. It should be emphasized, however, that the poor digestibility of the commercial zein must have been caused by the processing to which it was submitted. It is not a characteristic property of native zein.

Turning our attention to the other diet periods of this experiment, we observe that the N-retention and weight change values attained on the whole casein diet fall well within the

ranges previously found for the enzymatic digests of casein, lactalbumin, beef muscle and evaporated milk diets (Albanese and co-workers, '47). These were attained, it should be noted, in the absence of a cystine supplement. Indeed, the nutritional value of this diet was superior to that of the CTH-2 which was supplemented with L-cystine.

The poor nutritional quality manifested by the CTH-2 diet employed in this experiment is to be contrasted with that of the CTH diet tested in the earlier study. These diets differed in several respects: (a) in the present diet (CTH-2) a mixture of the synthetic B vitamins was substituted for the brewers' yeast of the CTH diet; (b) as noted above, the diets had a different physical consistency; and (c) the shortage of L-tryptophan forced us to use DL-tryptophan (in double quantity) in the preparation of diet CTH-2. Inasmuch as the subjects performed well on the casein diet, it is fair to assume that the poor quality of diet CTH-2 could not be attributed to a B vitamin deficiency or to poor assimilation because of its consistency. It remains, therefore, to question the use of DL-tryptophan as a physiological substitute for L-tryptophan. Evidence suggesting that this substitution may have untoward nutritional effects can be derived from the atypical brilliant red coloration obtained with the application of the Albanese-Frankston procedure ('45) for the determination of urinary tryptophan to the urine collected during the zein and CTH diet periods. This anomaly subsided almost immediately with the feeding of the casein or evaporated milk diets. Concomitantly, abnormally high values were obtained with the Shaw-McFarlane method (Berg and Rohse, '47) which were not apparently due to the excretion of D-tryptophan. The urinary tryptophan values as obtained by the microbiological procedure (Greene and Black, '44) were totally unaffected by the dietary changes. Oddly enough, negative tests were obtained with the urines of the zein and CTH period for the aberrant metabolite which we reported to be excreted by adults after the administration of DL-tryptophan (Albanese and Frankston, '44). This may be due to a differ-

ence in the relative amounts of tryptophan given, or to some differences in metabolic response between the adult and infant. It is obvious that further and more detailed studies are required for a complete evaluation of the utilization of D-tryptophan by man.

TABLE 4

Comparison of biological value of evaporated milk and corn gluten formulae in the normal male infant
(All results given as daily averages of 7-day periods)

SUBJECT	DIET	TOTAL N INPUT	DAILY BODY WEIGHT CHANGE	NITROGEN RETENTION	TOTAL PLASMA PROTEIN	DAILY FECAL N
		gm	*gm*	*mg/kg*	*gm %*	*gm*
	EM	3.31	+ 17	140	6.70	0.60
T.R.	CG	3.65	— 59	135	7.43	0.55
4.5 months	CG	3.68	— 34	26.5	6.80	1.47 [1]
6,370 gm	CG	3.53	— 5	65.5	7.50	0.51
	EM	3.45	+ 23	129	6.26	0.46
	EM	3.88	+ 23	139	7.20	0.34
	CG	3.23	0	98	7.00	0.38
A.J.	CG	3.81	— 66	100	7.65	0.24
6.0 months	CG	3.88	+ 3	48	7.68	0.72
6,837 gm	CG	3.90	+ 23	81	7.00	0.43
	EM	3.50	+ 17	121	7.11	0.57
	EM	4.00	+ 23	133	6.10	0.61
C.L.Y.	CG	3.98	— 27	116	6.53	0.53
6.3 months	CG	4.11	+ 15	60	6.10	0.53
7,107 gm	CG	4.17	+ 12	73	6.62	0.73
	EM	3.93	+ 12	132	6.10	0.52

[1] This high fecal N was caused by a diarrhea of cryptic origin.

Second experiment

Returning to the principal theme of this report, a nutritional evaluation of corn and wheat proteins, we next considered the possibility that the inferior biological value of the reinforced zein diet might be due to chemical changes induced in the zein during manufacture. It occurred to us, therefore, to assay corn gluten, a product which is subjected to less vigorous chemical treatment than zein. On the basis

of the experience already discussed, L-tryptophan instead of the racemate was used to correct the tryptophan deficiency of the protein. The results of this study, which are shown in table 4, disclose that this product, like zein, exhibited poor nutritional qualities. However, examination of the fecal N data discloses that this characteristic cannot be ascribed, as in the case of zein, entirely to poor digestibility. In the light of recent observations one is tempted to suggest that it may be due to an amino acid imbalance, a subject which has been

TABLE 5

Comparison of biological value of lysine-fortified gluten (WG) and evaporated milk (EM) formulae in the normal male infant
(All results given as daily averages of 7-day periods)

SUBJECT	DIET	TOTAL N INPUT	DAILY BODY WEIGHT CHANGE	NITROGEN RETENTION	TOTAL PLASMA PROTEIN	DAILY FECAL N
		gm	*gm*	*mg/kg*	*gm %*	*gm*
D.J. 3 months 4,064 gm	EM	2.81	+ 28	200	6.63	0.40
	EM	2.75	+ 28	182	5.81	1.35
	WG	2.74	+ 19	188	5.65	0.36
	WG	2.94	+ 24	188	6.21	0.34
J.A. 4 months 3,369 gm	EM	2.45	+ 37	162	6.63	0.40
	EM	2.53	+ 28	165	5.81	0.47
	WG	2.56	+ 22	179	5.65	0.42
	WG	2.67	+ 26	190	6.21	0.42
S.M. 3 months 3,230 gm	EM	2.46	+ 34	215	6.42	0.32
	EM	2.49	+ 23	222	5.96	0.29
	WG	2.35	+ 25	196	6.21	0.31
	WG	2.45	+ 28	195	6.18	0.57

recently reviewed by Elvehjem and Krehl ('47). A comparison of the amino acid composition of corn gluten and casein shows the principal difference to lie in the high leucine content of corn gluten (corn gluten 25%, casein 12%, Block and Bolling, '45).

Although this hypothesis is founded on surmise, supporting evidence can be adduced from the results of the bioassay of the lysine-reinforced wheat gluten diet, which show that diet WG (containing 12% leucine) supports nutritional

states in the infant comparable to those supported by the evaporated milk diet. Doubtlessly, numerous other factors may be responsible for the nutritional differences between the corn and wheat gluten diets, but the disparity in leucine content of the two products is an obvious variant. Because of the relative abundance and low cost of corn gluten, it would seem economically worthwhile to explore means of remedying its nutritional defects so that more complete utilization could be realized.

SUMMARY

It has been found that the nitrogen retention and weight gain of infants maintained on a synthetic diet in which tryptophan- and lysine-supplemented commercial zein constituted the principal source of nitrogen were inferior, by reason of the poor digestibility of the zein, to those obtained on a casein diet fed at the same fluid, caloric distribution and nitrogen levels. A diet in which tryptophan- and lysine-reinforced corn gluten provided the major source of protein also proved to be nutritionally inadequate for the infant. In contrast, a lysine-enriched wheat gluten diet supported a nutritional state in the infant comparable to that afforded by an evaporated milk formula. Evidence, albeit inconclusive, is presented which suggests that the inclusion of D-tryptophan in the diet may have an untoward nutritional effect.

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THE AVAILABILITY OF VITAMINS FROM YEASTS

V. DIFFERENCES IN THE INFLUENCE OF LIVE YEAST ON THE ABSORPTION OF PURE THIAMINE HYDROCHLORIDE, PURE RIBOFLAVIN AND NITROGEN BY HUMAN SUBJECTS, AND THE EFFECT OF DISTRIBUTION OF THE VITAMIN DOSES [1]

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Earlier experiments established that when live bakers' yeast is ingested by human subjects, not only the yeast thiamine but also food thiamine simultaneously consumed fails to be returned in the urine in normal amounts, presumably through failure of absorption (Parsons et al., '45). Reports by Bird et al. ('46) and Mims et al. ('47) of an inhibitor of the action of the vitamin B_c conjugase in crude yeast extracts suggested experiments to determine whether there might be a similar enzyme inhibitor in yeast which interferes with the availability of the thiamine from food in the diet and from fresh yeast, a variable part of which is in the combined form. This possibility appeared at first to be excluded, as it had

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already been shown (Hochberg et al., '45) that thiamine ingested in a certain sample of bakers' yeast in which 83% was in the free form was only 17% available to human subjects, and hence the unavailability does not depend necessarily on the occurrence of the thiamine in a combined form when ingested. However, inasmuch as yeast cells can be influenced to alter the form of their contained thiamine, as for example when certain conditions of drying induce a shift of some of the combined yeast thiamine into the free form (Melnick and Field, '39), one could not rule out arbitrarily the possibility that contact with digestive juices and other conditions in the intestinal tract might in some way induce the live yeast cell to convert its free thiamine to a form in which its release for absorption might be blocked by a yeast inhibitor.

Such an inhibitor then could conceivably account for the low availability of the thiamine, not only in the yeast of Hochberg et al. ('45) but also in the basal diet of Kingsley and Parsons ('47) in the presence of live yeast. In the latter case it would be assumed that only combined thiamine in the diet was being interfered with, and a mode of its encountering the hypothetical inhibitor in yeast could be postulated. Hence, in the present experiment, it was planned that so large a proportion of the thiamine intake accompanying the fresh yeast supplement should be in the free form that any interference with its absorption, if this were clearly shown, would require a modification of the hypothesis of an inhibtor stated above. Assays for riboflavin and nitrogen were planned in order to extend the basis for interpreting the nature of the influence of fresh yeast in the diet.

EXPERIMENTAL

The method of study was similar to that reported by Price et al. ('47), i.e., a basal diet [4] containing about 0.63 mg of thiamine and 3.7 to 4.2 mg of riboflavin per day (determined by weekly assay for thiamine by the thiachrome method of Hen-

[4] We are indebted to the Pineapple Research Institute of Hawaii for the gift of the specially packed crushed pineapple.

nessy, '47, and for riboflavin by the fluorometric method of Conner and Straub, '41) was ingested by normal college women over a 4-day period. The single test-day was preceded and followed by periods on the basal diet alone. As this short human bioassay was not suitable for determining nitrogen balances, the study of the availability of the protein of fresh yeast F was carried out with a diet squad maintained on different intakes for periods of three to 10 days (Ness et al., '46). Nitrogen was determined by macro-Kjeldahl assay.

Supplements included 5 mg of thiamine hydrochloride, 3 mg of riboflavin, 100 or 150 gm of a regular fresh live yeast prepared for the use of bakers (yeast F) and containing 4 to 7 μg of thiamine and 14 μg of riboflavin per gram (moist basis), and a preparation of non-viable yeast F. The yeast was killed by suspension in 70% ethanol for 48 hours and subsequent reduction to dryness before a fan in a darkened room. The viability of this alcohol-dried yeast was determined by plating various dilutions of the yeast suspension on a nutrient medium, and was found to be negligible.

The criterion of availability of thiamine and riboflavin from the yeast and from the supplements of pure vitamins used in this study was their urinary return as compared with that from positive control doses of 5 mg thiamine hydrochloride and 3 mg riboflavin given with the basal diet above but without the presence of yeast.

RESULTS

Results on thiamine

When free thiamine was given as the sole test dose with the regular meals, the urinary return was 22.4%. When free thiamine and fresh yeast were ingested together with meals, the urinary thiamine excretion was decreased to an average of 0.2% of the test dose (table 1). Two subjects of the 5 showed a decrease in urinary thiamine output below that on the basal diet alone. It can be concluded, then, that not only combined food thiamine is interfered with by fresh yeast ingestion, but

TABLE 1

Effect of presence of live yeast in diet and of time of intake of doses of thiamine and riboflavin on urinary return of vitamins

SUBJECTS	PERCENTAGE RETURN OF THIAMINE [1]			PERCENTAGE RETURN OF RIBOFLAVIN [1]		
	5 mg thiamine HCl alone		5 mg thiamine HCl with fresh yeast [1]	3 mg riboflavin alone		3 mg riboflavin with fresh yeast [1]
	Between meals	With meals	With meals	Between meals	With meals	With meals
FG	10.3	19.9	0	62	68	34
ZB	5.8	25.6	0 [2]	54	53	37
MP	15.8	28.6	0	53	64	39
PH	9.9	20.5	0.8	64	31	55
BC	20.6	17.5	0.3	48	64	42
Average	11.5	22.4	0.2	57	56	41

[1] Formula and method explained by Kingsley and Parsons ('47). When fresh yeast was included with the pure vitamin supplements, the total dose of thiamine was 5.4 mg and of riboflavin 4.4 mg.

[2] Lag day not carried out.

that viable yeast similarly affects the availability of thiamine hydrochloride ingested in pure solution.

It was desired to determine more clearly the effect of fresh yeast on the pure vitamin alone, without the presence of food. Hence, the supplements were given without the accompaniment of food and as far removed from meals as was convenient for the administration of doses in the laboratory. All subjects ingested 100 gm of yeast per test-day, divided in the following manner: 15 gm of yeast with each meal, and 55 gm of yeast plus 5 mg thiamine hydrochloride and 3 mg riboflavin in one dose between meals.

Urinary thiamine returns after the ingestion of free thiamine, plus that combined in fresh yeast taken in three doses with meals, averaged 0.2%. The average thiamine return from this same intake of the pure vitamin when ingested at one time, between meals with the fresh yeast, was 5.7% for 8 subjects, with a range of from 2.1% to 9.3%. This difference may be due not so much to the timing of the vitamin doses as to the allocation of too large a proportion of the yeast dose to the between-meal feeding of the pure vitamin, leaving too small an amount (15 gm) to accompany the meals and thus perhaps permitting more of the food thiamine to be absorbed by the digestive tract in the latter plan of feeding than in the former. In either case the simultaneous ingestion of fresh yeast with the thiamine supplement caused an unmistakable and striking depression of the urinary return below the level expected from ingestion of the vitamin alone.

It seemed desirable to test specifically the effects of the two methods of distribution of doses of the vitamins themselves without the accompaniment of yeast. The urinary returns of pure thiamine hydrochloride ingested as a single dose between meals averaged only 11.5% for 6 subjects (table 1), as compared with an average return of 22.4% for the same subjects ingesting the vitamin supplement with meals. Repeating this test with available thiamine in the form of dead dried yeast, distributed in the two ways, the average return for between-meal ingestion of the dose was only 13%, in contrast

to an average of 25.3% when the supplement was taken with the meals, thus closely conforming to the results obtained with the pure vitamin.

Melnick et al. ('39) observed a similar order of difference in urinary excretion of thiamine; the difference was greater when the positive control dose was taken with the heaviest meal of the day than when the subjects were in a post-absorptive and fasting state ". . . 12 hours after the last meal and 3 hours prior to breakfast." The same workers ('41) suggested that this trend of decreased urinary excretion following a test dose of thiamine hydrochloride may be due to one or both of two factors: the first postulation was that thiamine destruction is due to its instability in a relatively more alkaline bile and pancreatic juice in the post-absorptive state, when gastric secretion is at a minimum. Investigating the fate of thiamine *in vitro* in various digestive secretions, they found appreciable destruction ranging from 50% to 90% at pH 8.0 to 8.5 in the presence of bile or pancreatic juice. The second postulation was the possible absorption of the vitamin from the stomach during a relatively longer retention in that organ when the presence of food prolonged the emptying time.

The same contrast between returns of urinary thiamine which Melnick et al. ('39) demonstrated when thiamine was fed with the meal and 12 hours later, respectively, was almost exactly duplicated in the present experiments, when the interval after the meal was only two to two and one-half hours. Hence, the hypothesis of Melnick and his associates that this contrast is necessarily attributable to differences in the effects of a full stomach versus an empty, alkaline stomach is shown not to be tenable.

Results on riboflavin

Urinary riboflavin excretions following a 3 mg positive control dose of pure riboflavin taken with meals ranged from 31% to 77% for 13 subject-periods, averaging 54%. When fresh yeast containing 1.4 mg riboflavin per 100 gm dose

was ingested with the 3 mg riboflavin at meals, 5 subjects had an average return of 41% of the total dose, with a range of 34% to 55% (table 1). The availability of riboflavin from 100 or 150 gm supplements of the fresh yeast alone had been ascertained with 8 subjects; there was an average return of only 16% of the riboflavin content of the yeast, thus confirming earlier observations by Price et al. ('47). Fresh yeast contributed 1.4 mg or approximately one-third of the riboflavin of the total test dose when it was taken together with 3 mg pure riboflavin. Hence, considering the relative unavailability of riboflavin from fresh yeast and the range of individual variations in response to the positive control doses, it does not appear that live yeast interfered with the absorption of pure riboflavin when taken with meals.

In studies of supplements ingested between meals, 32% to 77% of the riboflavin from a 3 mg positive control dose was excreted in 17 subject-periods; the average was 57%. Six subjects were given the 3 mg pure riboflavin plus fresh yeast between meals. Returns ranged from 35% to 53%, averaging 46%. Therefore, contrary to results with thiamine, there was no striking difference in riboflavin returns whether the doses were taken with meals or between meals. Similar results were obtained when dead dried yeast, shown previously by Price, Marquette and Parsons ('47) to contain available riboflavin, was the supplement together with the pure riboflavin. The average return from between-meal ingestion of the dose was 52% for 10 subjects, and 53% when taken with meals by three subjects.

Results on nitrogen

Evidence is presented in table 2 that the protein of the fresh yeast cell is well absorbed by the human digestive tract. After a 6-day period on the basal diet alone, supplementation with 150 gm of fresh yeast F in a 6- or 10-day period for 6 subjects led to an average recovery in the urine of 80% of the 3.72 gm of nitrogen furnished by the yeast. This may be

TABLE 2

Comparison of urinary and fecal thiamine output with urinary and fecal nitrogen output on a basal diet with and without fresh bakers' yeast

PERIOD		NITROGEN ELIMINATIONS						GM/DAY		THIAMINE ELIMINATIONS	
		Individual subjects [2]						Avg.		Avg. μg/day [2]	
Type [1]	Length days	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces
Basal	6	9.70	1.42	9.78	1.05	9.69	1.27	9.72	1.27	312	680
Basal plus 150 gm fresh yeast F	6	12.98	1.55	12.46	1.52	12.75	1.64	12.73	1.57	98	1089
Basal	3	10.42	1.32	10.37	1.27	10.73	1.49	10.51	1.36	226	582
Basal	6	9.04		9.55		10.45		9.68		332	
Basal plus 150 gm fresh yeast F	10	13.07		11.66		13.0		12.58		68	
Basal	3	9.12		11.0		9.67		9.93		140	

[1] The basal diet contained 11.2 gm nitrogen; the 150 gm yeast F, 3.72 gm nitrogen.

[2] The two diet squads comprised three subjects each; fecal values are available for one squad only.

considered a high recovery in view of the fact that human subjects ingesting doses of fresh bakers' yeast in this laboratory return none of the thiamine of the yeast and have been repeatedly observed to eliminate 1 to 2% of the ingested yeast cells in the feces in a viable state (Parsons et al., '45) and as high as 28% in diarrheal feces (Stephenson, '45). Montgomery and Boor ('31) showed that under certain conditions as much as 95% of ingested fresh bakers' yeast cells were still viable as they left the human stomach; hence it could be assumed that considerable viability would persist in these cells in the intestinal tract, but, as is evident from the results of this experiment, not necessarily beyond the region where protein may still be efficiently digested and absorbed.

In the same 6 subjects (table 2) the characteristic drop in urinary thiamine output during the fresh yeast period was noted, i.e., a 74% average decrease from the level of output without yeast, indicating the unavailability of the yeast thiamine and an interference with food thiamine.

This difference in the effect of fresh yeast on urinary nitrogen and thiamine, respectively, was reflected to some extent in the different effects of the yeast on the two fecal values, in a general reciprocal relationship. The increase in nitrogen content of the feces (three subjects) during the yeast period represented only 8.5% of the nitrogen content of the fresh yeast supplement, whereas the 409 μg increase in fecal thiamine represented 44.5% of the thiamine content of the yeast dose. As might perhaps be expected, this increase in fecal thiamine did not quantitatively account for the drop in urinary output; the possibility of loss of thiamine in the digestive tract through such agencies as alkalinity (Melnick et al., '41) and intestinal flora must be taken into account.

DISCUSSION

The hypothesis that a simple anti-enzyme relationship is the essential factor in the interference by live yeast in thiamine absorption by human subjects is not substantiated by

the present experiment, in which it has been shown that pure thiamine hydrochloride is influenced in the digestive tract in the same manner as is food thiamine. It would appear to be more reasonable to postulate that the phenomenon involves an uptake of food thiamine by the live yeast cells analogous to the thiamine uptake from wort in the making of beer. Riboflavin is known not to be taken up appreciably by yeast from surrounding media such as wort, which would be in harmony with the observation that only the riboflavin of the live yeast cell, not that of the food or in pure form in the presence of live yeast, is relatively unavailable to the human digestive tract.

On the other hand, this hypothesis without further elaboration does not account for the strikingly higher utilization of nitrogen than of thiamine or of riboflavin from the fresh yeast cell. The true explanation is probably a more complicated one than either of the above hypotheses suggests and clarification must await further observations.

SUMMARY

A higher percentage of a dose of thiamine hydrochloride was returned in the urine of human subjects when the supplement was ingested with meals rather than between meals. The urinary return of riboflavin was not similarly influenced.

Pure thiamine hydrochloride ingested with fresh bakers' yeast failed to be returned in the urine, presumably through almost complete failure of absorption by the digestive tract. This was the same effect as that observed previously in respect to food thiamine ingested with live yeast. Hence, the "interference" by fresh yeast is not attributable per se to the effect of an anti-enzyme in the digestive tract.

In the same experiment live yeast interfered less with riboflavin than with thiamine in that only the riboflavin of the live yeast cell, not that in the food or in pure form, appeared to be withheld from absorption; nitrogen was least affected, inasmuch as an average of 80% of the nitrogen of the yeast cell itself was returned in the urine. That these

differences in excretion were actually due to differences in absorption tended to be borne out by fecal assays.

These observations make necessary some further elaboration of the hypothesis that food thiamine is interfered with by live yeast because of competition between the absorbing membranes of the live yeast cell and of the digestive tract, with a resulting retention of the absorbed thiamine within the yeast cell.

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THE DISTRIBUTION OF THIAMINE IN THE EMBRYONATED HEN EGG

I. THE CONTENT OF THE WHOLE EMBRYO [1]

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ONE FIGURE

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The thiamine content of the egg of the domestic fowl, as determined by the macro fermentation method, has been reported by this laboratory to remain essentially unchanged during incubation (Scrimshaw et al., '44). Before this work was completed, Westenbrink and Van Leer ('41) reported that most of the thiamine originally present in the egg fails to appear in the embryo. Their procedure differed from ours since they followed separately the drop of thiamine in the yolk and its increase in the developing embryo. Little is known of the role of thiamine in embryonic development, and the question of its utilization in the morphogenesis and maintenance of the chick embryo is of considerable interest. Therefore, it seemed necessary to investigate this apparent disagreement concerning the fate of the thiamine originally present in the egg. Accordingly, the procedure of Westenbrink and Van Leer of studying separately the thiamine in the embryo and in the extra-embryonic portion of the egg was followed in the present study.

The results of Westenbrink and Van Leer were not confirmed, although a small but significant loss of thiamine in

[1] Aided by a grant from Swift and Co., Chicago, Illinois.

the whole egg during development has now been demonstrated by using a larger number of eggs per sample and improved methods of preparation. This was obscured in the earlier investigation by the greater variation in the values and the failure to correct for the small total weight loss which occurs during incubation. This small drop in the total thiamine content is believed to represent the thiamine required for metabolic processes during development. However, in general, the assays carried out on these materials by the macro fermentation method and described below confirm the results previously published from this laboratory. Various bases for the above discrepancy are explored and a partial explanation is suggested.

MATERIAL AND METHODS

Ten eggs [2] of each series were selected at random and assayed as a single sample to determine a base-line thiamine value for the incubated group. The remainder were placed immediately in a standard electric incubator with fan and kept at temperatures of 38° to 41°C. and a humidity of approximately 40%. The eggs were turned twice a day.

Ten eggs were removed at the intervals listed and made up as a single sample. The eggs were weighed and then broken. The contents were then either weighed as a whole or separated so that the embryo and the remainder could be recorded separately. The "remainder" ordinarily consisted of all of the egg contents except the embryo and therefore included the yolk, allantois, amnion, and so forth. In a few specified cases, the eggs were divided into embryo, yolk, and remainder. These samples were then mixed in a Waring Blendor and diluted to a convenient volume. In the case of larger samples, an aliquot was weighed into a volumetric flask and diluted. All samples were then brought to a pH of 4.00 or below by the addition of HCl and stored in a cold room at about 5°C.

[2] The eggs were supplied from White Leghorn hens by Roger's Farms, a nearby commercial poultry farm and hatchery.

Samples thus prepared could be stored for several weeks without change in their thiamine activity.

All assays were carried out by the modified macro fermentation method (Scrimshaw and Stewart, '44). Duplicate runs were made whenever the quantity of sample permitted. Many values were checked by two separate cleavages and preparations of a sample and two runs in duplicate. Most samples were assayed at approximately the 2 μg level. All samples were heated for 45 min. in a boiling water bath in preparing them for assay, but no special digestion procedure was used. Autolysis at room temperature did not appear to change the thiamine content of acidified samples.

TABLE 1

Thiamine content of base-line samples of the series studied

SERIES	DATE	NO. IN SAMPLE	MEAN SHELL WT.	MEAN CONTENT WT.	B_1/EGG	B_1/100 GM
			gm	gm	μg	μg
V	10/12/44	10	6.7	50.3	48.3	96
VI	10/27/44	10	6.5	42.6	42.6	100
VII	1/4/45	20	6.7	47.6	44.3	93
VIII	1/29/45	10	7.4	51.8	37.3	72
IX	2/8/45	10	8.3	50.7	40.6	80
X	3/10/45	10	6.6	48.5	48.5	100
XI	4/12/45	10	7.3	50.6	50 6	100

EXPERIMENTAL RESULTS

Nine large lots of eggs were incubated during the fall of 1944 and the spring of 1945. The dates on which incubation was begun along with data on the base-line samples are shown in table 1. There is some suggestion that the lower values for series VII and VIII were associated with unusually severe winter weather at that time. The values obtained for the embryo and egg contents of the various samples are listed in table 2. When the egg and embryo thiamine values for all series are adjusted to an arbitrary base-line value of 100 μg per 100 gm of whole egg, they can be presented together.

The average thiamine content data are shown in table 2, but the sharp increase in thiamine content of the embryo and the relatively small loss of total thiamine in the egg are best shown in figure 1, where the individual sample values are plotted. Although the error in assay of late stages is relatively high, there is still evidence for a slight drop in thiamine content. This was obscured in the earlier study by factors indicated above. The curve for the increasing thiamine con-

TABLE 2

The thiamine concentration and total thiamine content of the developing embryo

AGE IN DAYS	EMBRYO				REMAINDER OF EGG			
	Concentration		Content		Concentration		Content	
	Actual	Corrected	Actual	Corrected	Actual	Corrected	Actual	Corrected
	μg/gm		μg		μg/gm		μg	
0	..	..	...	...	92 [1]	100 [1]	44.8 [1]	50.0 [1]
6	45	46	0.38	0.40	108	108	50.0	48.7
10	47	48	1.45	1.31	101	102	52.0	48.4
12	58	58	3.8	3.5	120	120	47.9	44.8
13	49	50	4.8	5.0	75	77	28.5	29.7
15	75	75	10.3	9.6	97	97	31.3	29.3
16	58	58	10.6	10.6		..	...	...
18	90	113	20.6	19.8	91	114	33.3	32.0
19	76	85	30.3	29.5	64	64	10.4	9.7
20	..	..	...	...	53	66	10.2	9.8
1-day chick	109	..	39.1	...	23	..	0.5	..

[1] Average 7 series.

tent of the embryo shows clearly that the bulk of the thiamine in the egg is not incorporated into the embryo until relatively late in the latter's development.

The tabulation of the total thiamine content of embryonic tissues reflects primarily the relative organ masses. The concentration of thiamine per gram in the various organs is a better indication of the importance of thiamine in their metabolism. In table 2 the rising thiamine concentration of the embryo is portrayed, along with the changes in thiamine

concentration of the remainder of the egg. Due to its minute size, we did not attempt to assay the embryo before the 6th day of incubation. The thiamine concentration appears to rise in a regular fashion in the embryo. It will be noted that the highest concentration of thiamine is found in the one-day-old chicks. On the other hand, there is no important change in the concentration of thiamine in the remainder of the egg until relatively late in development. The value for the remainder

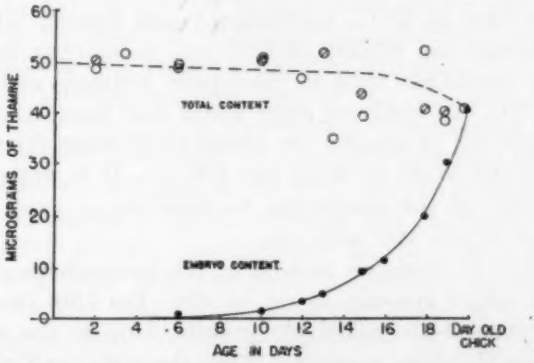

Fig. 1 The thiamine content of the whole egg and embryo during incubation. The small solid circles represent the content of the embryo at the various stages indicated. The large circles indicate the total thiamine content of the egg. The broken large circles represent values obtained by analysis of the entire egg contents. The clear large circles show the sum of the thiamine content of the embryo and non-embryo portions of the egg determined separately. All of these values have been corrected for initial variations in the thiamine concentration and weight of the eggs.

does not represent the thiamine concentration of the yolk. Although not included in the table, the initial concentration of thiamine in the yolk is approximately 300 μg per 100 gm; in the 18-day chick it has dropped to 120 μg; and as "spare yolk" the day-old chick contains about 20 μg per 100 gm. In the early stages all of the thiamine is contained in the yolk but as development proceeds the thiamine becomes widely distributed.

DISCUSSION

As noted above, all of the thiamine originally present in the egg is to be found in the yolk (Chick and Roscoe, '29; Bethke et al., '36; Ellis et al., '33; Scrimshaw and Stewart, '44). The distribution of this substance, like that of many others, must be regulated by the living yolk membrane (Needham, '31). The work involved in maintaining these tremendous concentration gradients is probably sufficient to account for most of the metabolic activity of the unfertilized egg (0.06 cal. per hour at 19°C., Langworthy and Barott, '21). After long storage the vitality of this yolk membrane is lost and osmotic gradients tend to disappear (Straub and Hooger-duijn, '29). In studying eggs which had been stored in the cold room for 16 months, we found an average thiamine content for the white of 40 μg per 100 gm. It is apparent that the ability of the membrane to maintain a steep thiamine gradient is also lost with age.

The loss in thiamine content of the non-embryo portion of the egg which appears to set in after the 15th day must in part represent the relatively greater drop in the volume of yolk (low thiamine content). That the yolk itself also drops in thiamine concentration is best shown by the figure for the remains of the one-day-old chick. Here the sample was obtained by dissecting out the "spare" yolk and the assay value represents yolk alone. This figure of approximately 20 μg per 100 gm is but 7% of the original concentration. Some of this drop undoubtedly occurred during the hours of muscular activity after hatching.

In a previous report (Scrimshaw et al., '44) the thiamine concentration per 100 gm of whole egg was discussed as if it were a direct reflection of the thiamine content of the egg. This is not strictly the case, although the variability of the data then presented did not warrant a discussion of it. It has long been known that there is a drop in the total weight of the egg as development proceeds. Tangl ('03) reported a loss of 17.9% although Murray's ('25) figure of 14.9% probably

represents a better average value. Our own series showed a weight loss of about 15% (8.1 to 16.5%). Unless the total concentration of thiamine at the end of development can be shown to be approximately 15% greater than that at the beginning of development, some loss of thiamine in the egg must have occurred. When cognizance is taken of the weight change the data show that a small loss has actually occurred. This is graphically illustrated by figure 1, and the loss appears to be approximately 20%.

This is a figure of interest because considerable respiratory activity can be measured during development (compare Romanoff, '41). In fact about one-third of the energy originally available in the egg has been used by the time of hatching. The relation of the energy used during development to that originally present in the egg, the apparent energetic efficiency (AEE), has been reported at 63 to 67 for the embryo chick (Tangl, '03; Murray, '26). If much of this energy exchange involved carbohydrate metabolism, as in non-embryonic forms, considerable thiamine would necessarily be required. However, the fact that the thiamine loss seems to be low in the developing chick embryo is consistent with the data of Needham ('31) showing fat to be the predominant energy source. The maintenance energy metabolism apparently involves a relatively small percentage of carbohydrate compared with the same respiratory exchange for the chick or adult. This is particularly true after the 10th day of incubation, when evidences of increased fat metabolism can be found (Murray, '26; Tallarico, '08; Riddle, '16). Before this time the total mass of the embryo is so small that its limited use of carbohydrate would not be reflected in our macro assays.

The results do not confirm the conclusion of Westenbrink and Van Leer ('41) that most of the thiamine originally present in the egg fails to appear in the embryo. In fact, most of the original thiamine is still present in the one-day-old chick which has had no opportunity to feed. The small loss which can be detected and attributed to developmental re-

quirements does not alter this general conclusion. It was considered possible that the eggs studied here contained so much more thiamine than the European eggs used by the above workers that the utilization of thiamine by the embryo was obscured. Study of their original paper when it became available showed that this was not the case, because all of the eggs used were similar to ours in thiamine content. Also, values obtained by us for incubated "low-thiamine eggs" refute this possibility.[3] The suggestion previously made that the discrepancy might lie in the extra-embryonic structures and fluids has not been borne out in the present studies. In the work presently reported, the thiamine content of the embryo doubled between the 16th and the 18th days and tripled between the 16th and 20th days. The actual content of the one-day chick was nearly double that of the 18-day embryo. It is apparent that most of the thiamine enters the embryo after the 16th day under the conditions of incubation in Rochester. If the 18-day embryo in Holland more nearly approximated the 16-day embryos of Rochester, the discrepancy in embryo thiamine content at this stage would not be great. We could then conclude that Westenbrink and Van Leer simply missed the great acquisition of thiamine by the embryo in the last few days of development. This would not, however, explain their report that the "eighteen day embryo plus yolk" contains only 35% of the amount initially present in the egg.

SUMMARY

The changing thiamine concentration of the embryo and the remainder of the egg contents has been determined by the macro fermentation method for embryonated eggs of White Leghorn fowls. The total thiamine concentration of

[3] Three hens were isolated and put on a diet containing less than 0.6 μg of thiamine per gram. The thiamine concentration of the eggs laid by these hens was determined. The average thiamine values for eggs laid on successive days of the experiment were 118, 97, 74, 72, 54, and 34 μg per 100 gm. Two eggs laid on the 7th day of the experiment were analyzed after 19 days of incubation and found to contain 35 μg per 100 gm.

the egg contents does not change greatly during incubation. However, by correcting for the loss of weight of the egg during development, a drop of about 20% is found and attributed to the needs of developmental metabolism.

The concentration of thiamine in the embryo gradually increases in an exponential fashion. The concentration in the remainder of the egg rises a little after the 7th day and begins to drop rapidly after the 15th day. The thiamine content of the remains as represented by the spare yolk of the day-old chick was found to have dropped to about 20% of the original concentration in the egg and about 7% of the original concentration in the yolk.

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THE INFLUENCE OF HIGH LEVELS OF FAT WITH SUBOPTIMUM LEVELS OF RIBOFLAVIN ON THE GROWTH OF CHICKS[1]

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THREE FIGURES

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The literature concerning the effect of fat in the diet on the riboflavin requirement is controversial. Potter, Axelrod and Elvehjem ('42) reported that lard had no effect on the riboflavin requirement of dogs fed sucrose diets. Mannering, Lipton and Elvehjem ('41) fed growing rats diets containing 25 to 40% "fat" and found that the riboflavin requirement increased with the fat level in the diet. In these experiments fat replaced dextrin. In a continuation of this work, Mannering, Orsini and Elvehjem ('44) concluded that the effect was due to the influence of the fat (lard and commercial hydrogenated cottonseed oil[2]) on decreasing the intestinal synthesis of riboflavin, since, when sucrose, which does not enhance intestinal synthesis, was substituted for dextrin the same reduced growth rate resulted. Tange ('41) has also reported an increased requirement of rats for riboflavin on high fat diets. Euler et al. ('42) have reported that the administration of linoleic acid to rats on a low riboflavin-low fat diet produced death in most animals.

[1] This work was carried on with the support of the Office of Naval Research under a contract with the Texas A. and M. Research Foundation.

[2] Crisco.

Since previous work on the requirements for riboflavin were with mammalia, it was of interest to extend studies of this nature to an avian species.

EXPERIMENTAL

The basal diet used had the following percentage composition: Wood pulp 3.0, cerelose 63.2, purified casein 18.0, gelatin 10.0, salt mixture 5.0, L-cystine 0.3 and fish liver oil 0.5. The vitamin supplement, in mg per 100 gm, was composed of thiamine hydrochloride 0.6, calcium pantothenate 2.5, choline chloride 250, nicotinic acid 7.0, pyridoxine hydrochloride 0.8, biotin 0.03, inositol 125, para-aminobenzoic acid 0.3, tocopherol 5.0, folic acid 0.25, 2-methyl-1,4-naphthoquinone 0.15. Fat was substituted for cerelose on a percentage basis. Not more than two weeks' feed was prepared at any one time, nor more than enough for three days' consumption placed in the feed troughs.

The chicks used were New Hampshire Red-White Leghorn crosses. In all experiments the chicks were distributed among the groups according to weights, so that there were the same number of light and heavy chicks in each group and the average weights in the groups were approximately the same.

In the first experiment (experiment I, table 1), two groups of 10 one-day old chicks were placed on the low fat and two groups on a 20% lard diet. One group on the low fat diet and one on the high fat diet were supplemented with 2 μg of riboflavin per gram, and the other two groups received 4 μg of riboflavin per gram of diet. Since the basal diet contained approximately 0.5 μg per gram, the total riboflavin contents were thus 2.5 and 4.5 μg per gram. These levels of riboflavin were selected because the 2.5 μg level is inadequate while the 4.5 μg one would normally meet the requirements for maximum growth.

The chicks were maintained on experiment for 40 days. At the end of that time the average gains of the chicks on the low riboflavin diets were 414 and 456 gm on the low and high fat diets, respectively. The average gains on the high ribo-

TABLE 1

The effect of fat in the diet on the growth of chicks receiving various levels of riboflavin

EXPERIMENT	GROUP	DIETARY REGIMENS		NO. OF CHICKS	AGE OF CHICKS		LENGTH OF EXPERIMENT	GAIN	STANDARD DEVIATION
		Fat	Riboflavin		Beginning	Termination			
			μg/gm		*days*	*days*	*days*	*gm.*	
I	1	None added	2.5	10	1	41	40	414	± 14
	3	20% lard	2.5	10	1	41	40	456	± 37
	2	None added	4.5	10	1	41	40	543	± 21
	4	20% lard	4.5	9	1	41	40	531	± 50
II	1	None added	0.5	12	14	33	19	65	± 5.1
	2	20% CSO[1]	0.5	10	14	33	19	5.8	± 2.7
	1	None added	4.5	9	35	47	12	228	± 17
	2	20% CSO[1]	4.5	9	35	47	12	210	± 15
III	1	None added	4.5	6	14	29	15	129	± 13
	2	20% CSO[1]	4.5	6	14	29	15	121	± 17
	3	None added	0.5	9	14	29	15	37	± 2.7
	5	20% lard	0.5	10	14	29	15	30	± 4.1
	6	20% Spry	0.5	10	14	29	15	26	± 3.2
	4	20% CSO[1]	0.5	10	14	29	15	3.1	± 2.1
IV	1	None added	4.5	9	14	27	13	162	± 11
	2	5% CSO[1]	4.5	10	14	27	13	169	± 11
	3	10% CSO[1]	4.5	10	14	27	13	174	± 12
	4	20% CSO[1]	4.5	10	14	27	13	177	± 16
	1	None added	0.5	9	27	39	12	140	± 11
	2	5% CSO[1]	0.5	10	27	39	12	90	± 10
	3	10% CSO[1]	0.5	10	27	39	12	64	± 15
	4	20% CSO[1]	0.5	10	27	39	12	51	± 8.1
V	1	None added	1.0	11	1	18	18	32	± 1.6
	4	20% CSO[1]	1.0	9	1	18	18	15	± 1.6
	2	None added	2.0	9	1	18	18	61	± 5.6
	5	20% CSO[1]	2.0	10	1	18	18	22	± 1.4
	3	None added	3.0	11	1	18	18	112	± 5.9
	6	20% CSO[1]	3.0	10	1	18	18	50	± 3.8

[1] Cottonseed oil. Wesson oil was used throughout.

flavin level were 543 and 531 gm on the low and high fat rations. The data analyzed statistically showed that there were no significant differences attributable to the lard.

An exploratory experiment (experiment II, table 1) was then performed, using a 20% cottonseed oil [3] diet instead of

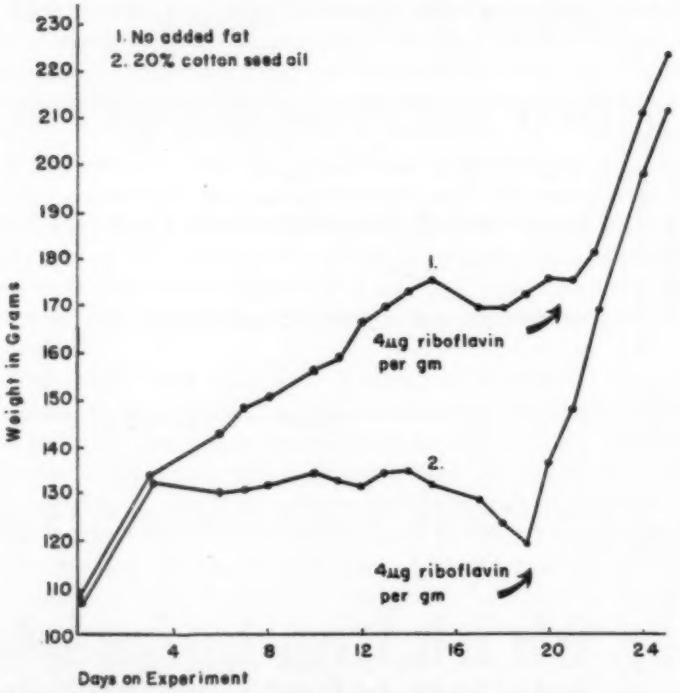

Fig. 1 Growth of 14-day-old chicks on low riboflavin diets containing 20% cottonseed oil and no added fat.

lard. The chicks had been on a stock diet for 14 days before being placed on the experimental ration. Only the fat content was varied, no riboflavin supplements being added for the first 19 days. Twelve chicks were started in each group. The results were strikingly different from those obtained on the

[3] Refined cottonseed oil available commercially as Wesson Oil.

experiment in which lard was fed. As may be noted in figure 1, the chicks on the cottonseed oil ration did not gain after the third day and began to lose weight sharply after the 14th day. The chicks on the low fat ration gained during the first 15 days and then failed to gain. Four micrograms of riboflavin per gram were then added to the cottonseed oil diet on the 19th day and to the low fat ration on the 21st day. The chicks subsequently gained rapidly, at about the same rate. As is seen in table 1, experiment II, on the 0.5 μg riboflavin level the low fat group gained 65 gm in 19 days, as compared to 5.8 gm for the cottonseed oil group. After supplementation with riboflavin the gains were 228 and 210 gm, respectively, in 12 days. This experiment indicated clearly a deleterious effect of cottonseed oil in a low riboflavin ration, which was corrected by the inclusion of riboflavin.

An experiment was then designed to confirm the observed responses of chicks to low riboflavin diets high in lard and cottonseed oil, and to test a hydrogenated vegetable oil. The effects of low fat and high cottonseed oil diets containing adequate riboflavin were also studied. The chicks were placed on experiment after 13 days on a stock diet. The results are presented in figure 2 and table 1, experiment III.

From figure 2 it may be seen that the low fat and high cottonseed oil groups of 6 chicks each, which received a supplement of 4 μg of riboflavin per gram (groups 1 and 2) grew at approximately the same rate. The low fat, lard and hydrogenated vegetable oil [4] groups of 10 chicks each which received no added riboflavin (groups 3, 5 and 6) gained slowly for 12 days and then began to lose weight. The cottonseed oil group of 10 chicks which received no added riboflavin (group 4) gained for only two days, maintained approximately the same weight for 10 days and then began to decline. On the 15th day 4 μg of riboflavin per gram was added to the riboflavin-deficient diets. The resulting gains were as great in the cottonseed oil group as in the others.

[4] Spry.

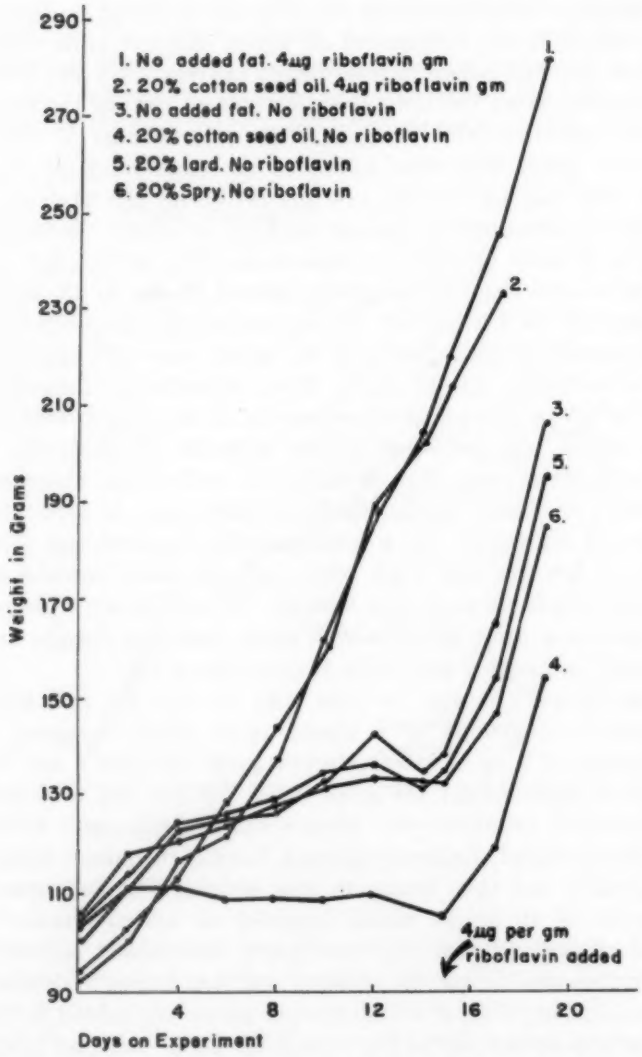

Fig. 2 Growth of 14-day old chicks on low and high fat and low and high riboflavin diets.

As shown in table 1, the average gain for the 15 days of depletion was approximately 30 gm by the chicks receiving lard, hydrogenated vegetable oil or no fat but was only 3 gm by those receiving cottonseed oil.

The deleterious effect of cottonseed oil which can be prevented or corrected by the inclusion of riboflavin is thus confirmed and contrasted to the comparatively innocuous effect of low fat, lard or hydrogenated vegetable oil rations.

To test the possibility that the development of rancidity might be responsible for the unfavorable effect of cottonseed oil, Nordihydroguaiaretic acid and citric acid were each added to this diet at a 0.05% level (experiment IV, table 1). Two-week-old chicks were used. Cottonseed oil was fed at levels of 5, 10 and 20%. Four micrograms of riboflavin per gram were mixed in the diets for the first 14 days and then discontinued. The average gains per chick on the 13th day were 162, 169, 174, and 177 gm on diets containing no fat and 5, 10 and 20% of cottonseed oil, respectively.

The riboflavin was then removed from the diets and on the 12th depletion day the respective average gains were 140, 90, 64 and 51 gm per chick on the low fat and 5, 10 and 20% cottonseed oil diets. Thus the protective role of riboflavin was again confirmed, and the deleterious effects of cottonseed oil shown not to be due to rancidity. Furthermore, the higher the level of cottonseed oil in the diet, the lower the rate of growth (fig. 3).

Since in all experiments with cottonseed oil the chicks had been placed on experiment after two weeks on a stock diet, it was decided to test the effects of cottonseed oil starting with day-old chicks. Three levels of riboflavin, 1.0, 2.0, and 3.0 μg per gram of feed, were used with low fat and 20% cottonseed oil diets. The growth data are summarized in table 1, experiment V. The better growth of the low fat groups is obvious.

The chicks receiving diets containing 20% of cottonseed oil with all three levels of riboflavin made less efficient gains than the chicks on the diets with no added fat. The grams of feed required to produce 1 gm of gain on the 20% cotton-

seed oil diets were 3.37, 2.90 and 2.04, respectively, for the 1, 2 and 3 μg levels of riboflavin. The feed consumed per gram of gain by chicks on the diets with no added fat for the corresponding riboflavin levels were 2.71, 2.21 and 1.36 gm. On the basis of the energy content of the diets, the high fat diets might have been expected to give the most efficient gains.

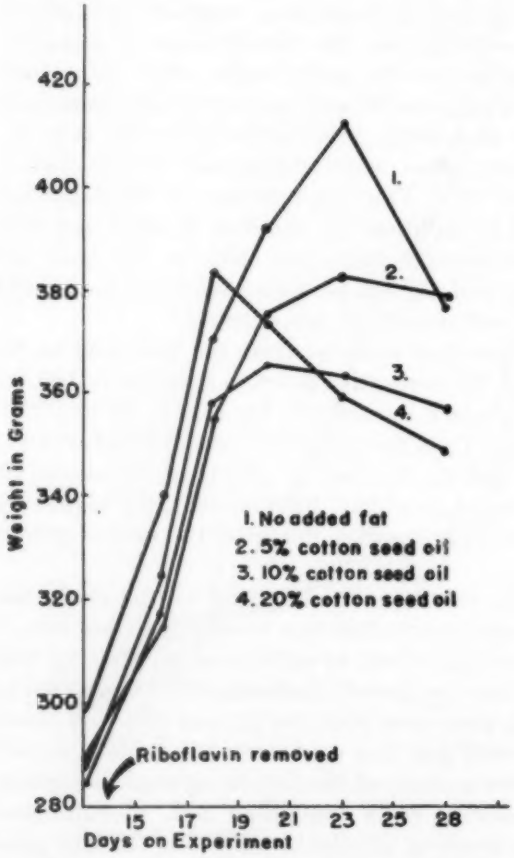

Fig. 3 The effect of graded levels of cottonseed oil on the growth of 14-day-old chicks upon removing riboflavin from the diets.

On the 18th day of the experiment the chicks were sacrificed and the livers of those receiving 1 and 3 μg of riboflavin per gram of diet assayed for riboflavin by the method of Roberts and Snell ('46). The riboflavin content of the livers was not affected by the addition of fat to the diets. On the 1 μg level of riboflavin, the average riboflavin content of the livers on the low fat diet was 7.2 μg per gram of fresh liver, as compared with 7.8 μg for the chicks on the diet containing 20% of cottonseed oil. On the diets containing 3 μg of riboflavin, the average levels in the liver were 15.9 on the low-fat diet and 13.7 on the diet containing cottonseed oil. The lower riboflavin content of the liver on the low levels of intake is in accord with the finding of Clandinin ('46).

The pronounced inhibitory effect on growth and the inefficient use of feed resulting from the inclusion of 20% cottonseed oil in a diet containing a suboptimum amount of riboflavin are again clearly demonstrated.

DISCUSSION AND SUMMARY

Chicks receiving moderately high levels of refined cottonseed oil in diets low in riboflavin cease growing much sooner than those on a similar diet that contains no added fat. When 4 μg of riboflavin per gram of feed are added to the diets, the growth response is essentially the same irrespective of whether the diets contain fat. Lard did not retard the rate of growth, nor did a commercial hydrogenated vegetable fat.[5] The response to cottonseed oil does not appear to be due to the development of rancidity since means were taken to prevent its formation, including the addition of anti-oxidants and refrigeration of the feed.

The chemical difference between cottonseed oil on the one hand and lard and hydrogenated vegetable oil on the other is the high concentration of linoleic acid in the former. Since there is no known reason for believing that the unsaturated acids require more riboflavin for their metabolism than the

[5] See footnote 4, page 251.

saturated it would seem more probable that the unsaturated acids interfere more with the intestinal synthesis of riboflavin.

The alternative possibility, however, must still be considered. Lehninger has recently shown ('48) that cytochrome is an essential constituent of the enzymatic system for the oxidative catabolism of fatty acids. Since riboflavin is a constituent of the enzyme system which catalyzes the reduction of the oxidized form of cytochrome C (Haas, Horecker and Hogness, '40), it becomes apparent that this vitamin has a role in fatty acid catabolism and an increased requirement might be expected on a high fat diet.

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EFFECT OF FAT IN THE DIET OF RATS ON THEIR GROWTH AND THEIR EXCRETION OF AMINO ACIDS [1]

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The importance of the interrelationships among the various nutrients of the diet has been increasingly realized during the last decade. Among these relationships, dietary fat has been found to influence energy and protein metabolism and various physiological functions. Increasing the fat content of isocaloric diets from 2 to 30% resulted in an increase in the rate of weight gain and a decrease in the heat production of rats (Forbes, Swift, Elliott and James, '46; Forbes, Swift, James, Bratzler and Black, '46). Rats fed diets low in protein made more rapid and efficient gains when 30% of fat was included in the diet than when only 2% was included (French, Black and Swift, '48). Deuel et al. ('47) have shown that feeding rats diets containing from 5 to 40% of fat results in better growth, greater physical capacity, and better reproductive and lactation performance than does the feeding of a low fat diet supplemented with methyl linoleate. The low fat diets used in previous investigations reviewed by the present authors were provided with the essential unsaturated fatty acids, so the beneficial effects of the fats must be attributed to other factors. Annegers and Ivy ('47) have shown that increasing the fat content of the diet delays gastric

[1] This work was carried on with the support of the Office of Naval Research under a contract with the Texas A. & M. Research Foundation.

evacuation. This might favor more complete digestion of the various nutrients.

The experiments reported here were designed to examine the effect of fat in the diet on the utilization of the protein component of the diet under conditions of isocaloric feeding and ad libitum feeding. The criteria used were rate of gain, gain per gram of protein consumed, and the fecal and renal excretion of phenylalanine, valine, lysine and methionine. The rationale of the latter was the finding that the amounts of amino acids excreted in the urine are influenced by the quality of the protein ingested (Sauberlich, Pearce and Baumann, '48). With proteins of high biological value the urinary excretion of amino acids was much lower than when proteins of a low biological value were fed. These findings indicate that one means of measuring the efficiency of utilization of proteins is by the renal excretion of amino acids. The size of the three-day sample of urine and feces did not permit assays for all of the essential amino acids.

EXPERIMENTAL

The percentage composition of the fat-free (low fat) diet was: purified casein 20, sucrose 76 and salts mixture 4. The fats used in these experiments were lard and a refined corn oil. The components of the diets containing fat were purified casein 22%, sucrose 66%, salts mixture 4% and either corn oil or lard 8%. One gram of the low fat diet was calculated on the basis of physiological heat values for protein, fat and carbohydrate to provide energy and protein equivalent to 0.906 gm of the diets containing fat. Vitamins were added to the diets in mg per 100 gm of diet as follows: tocopherol acetate 0.5, vitamin D_3 0.2, vitamin A 0.2, 2-methyl-4-naphthoquinone 0.1, thiamine 0.25, riboflavin 0.3, pyridoxine 0.25, Ca-pantothenate 2, choline chloride 100, niacin 0.5, inositol 100, pteroylglutamic acid 0.2 and biotin 0.01. The diets were stored in a refrigerator. Each rat was given approximately 30 mg of ethyl linoleate daily by means of a medicine dropper. This is adequate to meet the needs of the rat for the essential

polyunsaturated fatty acids. The ethyl linoleate was given to the rats receiving the diets containing fat to insure as much uniformity as possible in the treatment of the animals.

White rats of the Sprague-Dawley strain were used. To avoid variations due to sex differences, only male rats were used. The details of the method of feeding will be given for each series as the results are discussed.

Quantitative collections of the urine and feces were made for three consecutive days. The period of collection of feces and urine was between the beginning of the third week and end of the 4th week, unless otherwise indicated. It was necessary to stagger the collections over the two-week period. An equal number of rats from each group were always in the metabolism cages at the same time. The urines were filtered and stored in a refrigerator. The feces were dried and ground for analyses.

Microbiological assays for the amino acids in the feces and urine were made on the hydrolyzed samples and represent, therefore, the total content of phenylalanine, valine, lysine and methionine. Methionine and lysine were determined with the test organism *Leuconostoc mesenteroides* P-60, using the procedure of Lyman et al. ('46). Valine and phenylalanine were assayed with *L. arabinosus* (Schweigert et al., '44) with the modification that 0.02 μg folic acid and 1 μg pyridoxamine were added per 10 ml of medium.

Ad libitum feeding

In this series the rats were fed the three diets ad libitum in individual cages, thereby permitting individual feed consumption records. There were 12 male rats in each group with an initial weight of approximately 40 gm. The gains for the 4-week period and gain per gram of protein consumed, together with the standard deviations, are shown in table 1. The rats fed the diet low in fat made an average gain of 76 gm, as compared with 107.8 gm for the group receiving corn oil in the diet and 105.2 gm for the group with lard in the

diet. The difference in the gains made by the group fed the low fat diet and the groups fed the diets containing 8% of fat was highly significant statistically. While the rats receiving 8% of fat in the diets made more gain per gram of protein consumed than the group fed the low fat diet, these differences are not statistically significant. The corn oil and lard groups consumed more feed than the low fat group and the lower food consumption is without doubt a factor in the less rapid growth of the latter group. The superior growth performance of the rats fed diets containing fat is in accord with previous observations (Deuel et al., '47; Scheer et al., '47).

TABLE 1

Growth of rats on diets containing corn oil, lard and no fat

DIETARY REGIMEN	GAIN IN 4 WEEKS		GAIN PER GRAM PROTEIN CONSUMED	
	Mean	S.D.[1]	Mean	S.D.[1]
	gm		*gm*	
No fat	76.0	17.3	1.4	0.3
Corn oil	107.8	15.1	1.6	0.3
Lard	105.2	15.8	1.7	0.3

[1] Standard deviation.

Excretion of amino acids

This series was designed to measure the effect of fat in the diet on the renal and fecal excretions of phenylalanine, valine, lysine and methionine. Data were also obtained on growth and efficiency of utilization of feed. There were 15 male rats in each group with an initial average weight of approximately 42 gm. Each animal was fed in an individual cage and an accurate record was kept of the amount of food consumed. The allowance of food was such that each animal in all groups received essentially the same amount of protein and energy. That this was essentially achieved among the three groups is evidenced by the fact that the average amounts of protein consumed per rat for the 4-week period showed a maximum difference of only 0.2 gm. The urine and feces

were collected separately over a three-day period. The number of metabolism cages available and the time sequence did not permit obtaining excretion data on all of the 15 animals in each group. Collections were made on 12 rats in each of the three groups. In a few cases the size of the sample of feces or urine was not sufficient to permit assays for all of the 4

TABLE 2

Effect of fat in the diet of rats on their growth and their excretion of amino acids

CATEGORY OF INTEREST	DIETARY REGIMEN					
	No fat		Corn oil		Lard	
	Mean	S.D.[1]	Mean	S.D.[1]	Mean	S.D.[1]
Gain/4 weeks (gm)	57.4	5.7	61.7	6.1	60.2	6.1
Gain/gm protein consumed (gm)	1.7	0.1	1.8	0.1	1.8	0.1
Phenylalanine excreted/3 days in:						
urine (mg)	2.0	1.4	1.3	0.6	1.3	0.8
feces (mg)	4.9	1.7	4.1	2.3	4.3	1.6
Valine excreted/3 days in:						
urine (mg)	1.7	1.3	0.9	0.03	1.5	0.2
feces (mg)	10.2	3.0	7.0	1.4	8.7	2.1
Lysine excreted/3 days in:						
urine (mg)	2.4	1.2	1.8	0.6	2.0	0.8
feces (mg)	8.6	3.3	6.4	1.9	7.5	1.7
Methionine excreted/3 days in:						
urine (mg)	0.64	0.4	0.4	0.1	0.5	0.3
feces (mg)	3.0	0.9	2.1	0.6	2.5	0.9

[1] Standard deviation.

amino acids. The growth data in table 2 and the gain per gram of protein consumed are for 15 animals in each group, while the figures for amino acid excretions represent either 11 or 12 animals in each group.

The mean gain in weight of the rats fed the low fat diet for the 4-week period was 57.4 gm, as compared to 61.7 and 60.2 gm for the groups receiving corn oil and lard, respectively. These differences are not significant statistically.

The amounts of amino acids excreted in the feces were from two to 5 times greater than were excreted by the renal pathway. We have no data to indicate the proportion of the fecal amino acids that comes from ingested protein. It is probable that a substantial proportion originates from other sources. On the basis of work with humans Sheffner, Kirsner and Palmer ('48) have estimated that fecal amino acids represent to a large extent amino acids secreted into the lumen of the gastrointestinal tract as components of digestive enzymes and excreted as such or converted into bacterial protein before elimination from the body. Nevertheless, it may be assumed where significant differences occur that they are not artifacts, since amino acids of endogenous origin would not be expected to differ essentially with the various dietary regimens.

The fecal excretions of each of the 4 amino acids were less by the rats receiving corn oil in their diet than by the rats receiving the diet with no added fat. Similarly, the amounts of each of the 4 amino acids excreted in the feces were less on the diet containing lard than on the basal diet containing no fat. There was a tendency for the fecal excretion of the amino acids to be lower on the corn oil diet than on the lard diet, but most of these differences are of small magnitude and it is doubtful if much significance can be attached to them. Statistically the difference between the fecal excretion for the low fat diet and the corn oil diet was significant for lysine, highly significant for valine and methionine, and not significant for phenylalanine. One interpretation of this is that corn oil at a level of 8% in the diet favors the digestion and absorption of lysine, methionine and valine.

The average renal excretion by the rats receiving corn oil was 0.9 mg of valine and 0.4 mg of methionine for the three-day period. The corresponding figures for the rats not receiving fat in their diet are 1.7 and 0.6 mg, respectively, for valine and methionine. Differences in the urinary excretion of these two amino acids are significant statistically, while for phenylalanine and lysine the differences are not statisti-

cally significant. These results show that 8% of corn oil in the diet of rats enhances the efficiency with which methionine and phenylalanine are utilized by this animal.

Tissue replenishment

This series was designed to measure the rate of tissue replenishment in rats fed a diet with no added fat and a diet containing 8% of corn oil. Rats that had been fed a stock

TABLE 3

Effect of fat on the rate of weight gain and the excretion of amino acids during tissue replenishment

CATEGORY OF INTEREST	DIETARY REGIMEN			
	No fat		8% corn oil	
	Mean	S.D.[1]	Mean	S.D.[1]
Gain/3 weeks (gm)	76.1	7.6	78.2	8.5
Phenylalanine excreted/3 days in:				
urine (mg)	5.5	2.4	5.3	3.7
feces (mg)	7.5	2.4	7.7	2.8
Valine excreted/3 days in:				
urine (mg)	8.1	4.5	6.6	3.3
feces (mg)	26.9	10.7	28.5	16.1
Lysine excreted/3 days in:				
urine (mg)	8.5	3.1	7.5	2.3
feces (mg)	11.9	3.0	10.9	4.0
Methionine excreted/3 days in:				
urine (mg)	2.8	1.36	2.3	1.2
feces (mg)	6.0	1.85	5.6	2.3

[1] Standard deviation.

ration until they weighed approximately 215 gm were then placed on a diet consisting of sucrose, salts, and vitamins at twice the levels used in the diets containing protein. During the depletion period each rat received 30 mg of ethyl linoleate daily. After they had lost between 30 and 40% of their weight they were divided into two groups of 10 rats each and placed in individual cages. The rats receiving the diet containing 8% of corn oil and those fed the diet containing no added

fat were fed at levels to provide equal protein and calorie intakes. They were kept on the experimental diets for three weeks, at which time their rate of gain reached a plateau. Between the 10th and 21st day of the experiment the urine and feces were collected individually for a three-day period.

The data on the rate of gain and the excretion of 4 amino acids are shown in table 3. The differences between the two groups with respect to rates of gain and the excretions of phenylalanine, valine, lysine and methionine were not significant statistically. It should be noted, however, that there is a tendency to excrete less amino acids when corn oil is included in the diet than when no fat is added to the diet. There was less lysine and methionine excreted in the feces by the rats receiving corn oil in their diet than by the rats receiving the diet with no added fat. The average renal excretion of each of the 4 amino acids was less by the group receiving corn oil than by the group with no added fat in the diet. The results using the replenishment technique are in the main in accord with the observations with young rats.

SUMMARY

Rats fed ad libitum a diet with no added fat and supplemented with ethyl linoleate gained 29% less than rats fed diets containing 8% of corn oil or lard. There was no significant difference in the gains of rats when the intakes of protein and energy were equalized.

The fecal excretion of phenyalanine, valine, lysine and methionine was from two to 5 times greater than the excretion by the renal pathway. The fecal excretions of the 4 amino acids were less by the rats receiving corn oil in their diet than by rats receiving a diet with no added fat. The differences were statistically significant for valine, lysine and methionine. The urinary excretion of valine and methionine was significantly less by the rats fed the diet containing corn oil than by the rats fed a diet containing no added fat.

Mature rats that had been fed a depletion diet until they had lost approximately one-third of their weight were fed on

a regimen providing equalized intakes of protein and energy. One group was fed a diet containing 8% of fat and the other group a diet with no added fat. The results, on the basis of growth and excretion of amino acids, favored the diet containing corn oil. The differences, however, were small and not statistically significant.

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THE DISTRIBUTION OF THIAMINE IN THE EMBRYONATED HEN EGG

II. THE CONTENT OF EMBRYONIC TISSUES [1]

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ONE FIGURE

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Despite the great amount of embryological and biochemical work done on the chick embryo, studies of the vitamin content of the embryonic tissues are rare. Barnett and Bourne ('41) have studied the distribution of ascorbic acid in the embryo during the first 4 days of incubation. The concentrations of 8 of the vitamins of the B complex, including thiamine, have been determined by Williams et al. ('41) for the liver, brain and hearts of 12-day embryos and day-old chicks. Attempts to modify these values by changing the diet of the hen or injecting vitamins into the egg have been reported from the same laboratory (Taylor et al., '41).

Studies of the thiamine content of the entire egg and intact embryo have been discussed in a preceding paper (Scrimshaw, Porter and Scrimshaw, '49). In this report the thiamine concentration and content of the embryo were shown to increase in exponential fashion. The thiamine concentration of the egg does not change until it drops rapidly during the final two or three days of incubation. The total thiamine content of the egg appears to drop about 20% by the time of

[1] Aided by a grant from Swift and Co., Chicago, Illinois.

hatching. The yolk contains all of the thiamine present in the egg before incubation (compare Chick and Roscoe, '29; Ellis et al., '33; Bethke et al., '36). The concentration of the yolk thiamine falls from approximately 300 μg per 100 gm at this time (Scrimshaw and Stewart, '44) to 23 μg per 100 gm for the "spare yolk" of the day-old chick.

In the present study the distribution of thiamine in the 18-day chick has been studied by assay of brain, liver, muscle, heart, gastrointestinal tract, kidney, yolk, membranes and remainder. The vitamin content of certain of these organs has also been studied in the 15-day embryo and the day-old chick. These values are correlated with those previously reported for the embryo and the whole embryonated egg.

MATERIALS AND METHODS

The eggs [2] were incubated as described previously (Scrimshaw, Porter and Scrimshaw, '49). Thirty eggs of the desired age were usually selected and the embryo, yolk and remains carefully separated and weighed. The embryos were then dissected. Each kind of organ or tissue was then weighed and prepared as a single sample. Most of the samples were mixed immediately in a Waring Blendor, acidified to pH 4.00 or below with HCl and diluted to volume for storage at 5° C. Striated muscle samples were placed in a vacuum desiccator in the cold room and ground up in a mortar when dry. The powdered samples were then treated as above.

The assays were done by the modified macro fermentation method (Scrimshaw and Stewart, '44). Often the quantity of sample was too small to permit duplicate runs. Where a value has not been checked by either a duplicate assay or by the assay of the same organ in a different series, this is indicated in table 2. Each of the 18-day values is the average of several series. Only one series of 15-day embryos and one-day-old chicks were studied.

[2] The eggs and day-old chicks were supplied from White Leghorn hens by Roger's Farms, a nearby commercial poultry farm and hatchery.

RESULTS

The distribution of thiamine in the embryonated 18-day egg is described first because it received the most complete study and can be used as a basis for comparison. In table 1 the mean thiamine concentration and range of values obtained for each of the egg components tested are given, together with the mean weight of each in an individual egg. It will be noted that the concentration in the liver is approximately 4 times

TABLE 1

The concentration of thiamine in the tissues and organs of 15-day embryos and day-old chicks

STRUCTURE	NO. OF SAMPLES ASSAYED [1]	MEAN WT. PER EGG	CONCENTRATION [2]	
		gm		*μg/gm*
Brain	3	0.78	1.50	(1.42–1.58)
Liver	3	0.45	3.68	(3.40–4.05)
G.I. tract	2	1.66	0.80	(0.35–1.25)
Kidney	2	0.14	1.95	(1.78–2.12)
Heart muscle	4	0.17	0.82	(0.56–1.34)
Leg muscle	2	...	1.35	(1.13–1.57)
Yolk	3	15.0	1.12	(1.05–1.19)
Membranes and fluid	2	12.6	0.92	(0.23–1.60)
Total less embryo	1	29.2	0.91	
Total embryo	2	18.6	0.91	

[1] These are the number of separate samples made up of 20 to 30 embryos assayed in duplicate.

[2] Values are not corrected for variations in initial thiamine concentration because such adjustment would not greatly affect the gross range of values.

that in the whole embryo. Kidney and brain and skeletal muscle are also seen to be higher in thiamine concentration than the mixed whole embryo, while the heart is about the same. It is of interest that at 18 days of incubation the concentration of thiamine in the embryo, in the yolk, and in the remaining fluid and membranes is approximately the same.

When these data are compared with those for the embryo at 15 days and the newly hatched chick presented in table 2, certain characteristic differences and similarities are noted.

There appears to be no significant change in the heart thiamine concentration. The concentration in the brain seems to drop steadily with development. By the time of hatching, a definite drop in the thiamine of the liver, muscle, gastrointestinal tract and yolk has occurred. In general, the values for 15 days do not differ markedly from those for 18 days except for a previously noted increase in concentration of thiamine in the whole embryo with age.

TABLE 2

The concentration of thiamine in the tissues and organs of 15-day embryos and day-old chicks

STRUCTURE	15-DAY EMBRYO		1-DAY CHICK	
	Mean weight	Concentration	Mean weight	Concentration
	gm	*μg/gm*	*gm*	*μg/gm*
Brain	0.587	2.50 [1]	0.895	1.18 [1]
Liver	0.290	3.34	1.23	2.58
G.I. tract	...	...	4.7	0.63
Heart	0.127	0.87 [1]	0.295	0.96 [1]
Leg muscle	...	...	...	0.84
Whole embryo	13.7	0.75	36.2	1.09
Yolk	32.3	0.97 [2]	2.0	0.23

[1] Only a single assay of a 30-embryo sample was done because of the limited quantity of material. Other values represent duplicate assays of a single sample. Values are not corrected for variations in initial thiamine concentration.

[2] Includes entire non-embryo portion of egg contents.

The average initial concentration of thiamine varied from one series to the next, as did the mean initial weight of the egg contents.[3] The cumulative effect of these differences would so distort the graphical presentation of the organ thiamine contents that these values are corrected for this variation. One hundred micrograms of thiamine per 100 gm of whole egg and an average weight of 50 gm per egg were values close enough to the true averages to serve as convenient arbitrary standards. If the initial content of a series averaged 90 μg of thiamine per 100 gm, individual tissue

[3] The average initial values for each series are given in the preceding paper.

values of this series were multiplied by 100/90. Similarly, an average initial egg content weight of 45 gm would result in all mean organ weights for the series being multiplied by 50/45. The adjusted results are plotted in figure 1. It will be seen that in general the content increases with development due to the increase in mass of the organ or tissue studied. Changes in concentration with development are not great enough to obscure this trend. This is particularly noticeable in the case of the liver and the gastrointestinal

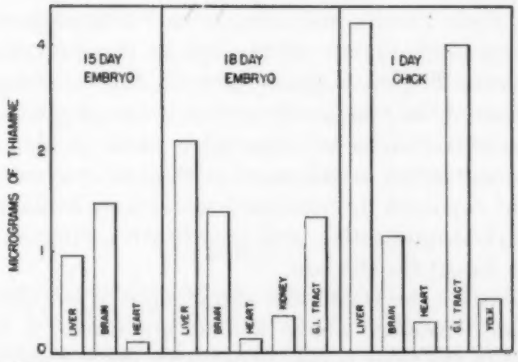

Fig. 1 Thiamine content of certain organs of the 15-day embryo, 18-day embryo and day-old chick. These values, corrected for initial variation in weight and thiamine concentration of the various series (see text), give the total thiamine to be found in the "average" organ at the stage indicated.

tract, which dropped considerably in concentration in the newly hatched chick but which later contained relatively large amounts of thiamine. Only in the case of the brain and, of course, the yolk, is a drop in thiamine content with development noted. If it had been possible to estimate the total mass of skeletal muscle, its thiamine content would undoubtedly have been the highest of any single tissue.

DISCUSSION

The single value reported by Williams, Taylor and Cheldelin ('41) for the liver of the 12-day embryo is two to three

times the one obtained by us for the 15-day embryo. However, their values are expressed in terms of dry weight and can be only approximately compared. Their figure for the liver of the day-old chick is in good agreement with ours. A single figure reported by Williams, McMahan and Eakin ('41) in a concurrent study was 1.9 μg per 100 gm of fresh "chick" liver.

The concentration of thiamine in both the heart and brain of the 12-day chick embryo as reported by Williams et al. ('41) is greater than that found by us in the 15-day embryo. However, their results and ours seem reasonably consistent except for a much higher value cited by them for the 12-day embryo liver. The discrepancy may be due to a higher thiamine content of the younger liver, but it can also be accounted for by the variation to be expected in their single sample.

The concentration of thiamine in the embryo, weaned, and mature rat reported by the above workers in the same study shows marked qualitative and quantitative differences from the values found for the hen.

In some cases the functional significance of the distribution of thiamine described above will be apparent. A high concentration in the liver is consistent with the metabolic importance of this organ. Similarly, nervous tissue and kidney might be expected to show a high thiamine concentration. The higher thiamine value for the 18-day chick muscle as compared with that of the young chick is not so easily understood. Perhaps the considerable muscular activity of the young chicks during and after hatching had depleted the thiamine stored before this time.

The steady drop in the thiamine content of the yolk is of interest since by the time of hatching the total thiamine remaining is only one-sixth of that in the gastrointestinal tract to which the yolk is attached. In fact, it has only slightly more thiamine than the much smaller heart. Although originally containing all of the thiamine in the egg at a concentration 4 times that of the final embryo, the yolk has lost all significance as a thiamine store by the time of hatching.

The changes that take place in the embryo during the last three days of development are great. At 18 days the embryo still makes up only about half of the total weight of the egg contents and a corresponding proportion of the thiamine. However, the newly hatched chick has incorporated much of this 50% of both thiamine and tissue. These changes which take place during the final days of incubation are difficult to study because of the variation among individual embryos and technical difficulties in handling samples with much foam and feathers. Considerable additional work is required on these critical final days.

Although certain trends can be noted in the above data with respect to the thiamine content and concentration of embryonic organs and tissues, detailed analysis must await further study. Indeed, only with increased knowledge of the role of thiamine in development and metabolism can these results be satisfactorily interpreted.

SUMMARY

The thiamine concentration and content of the organs and tissues of 15-day and 18-day chick embryos, and of day-old chicks, were determined by the macro fermentation method. In the 18-day embryo the liver showed the highest concentration (4.5 μg/100 gm), followed by kidney (2.4), brain (1.8), muscle (1.8), gastrointestinal tract (1.0), and heart (1.0). The embryo at this stage showed a concentration of 1.1, the yolk 1.5, with the membranes and fluids intermediate. The corresponding values for the 15-day embryo are similar but those for the one-day chick are generally lower.

The total content is greater in the brain at 15 days than in the liver (1.5 as compared to 1.0 μg). By 18 days the liver contains the most thiamine (2.0 μg), followed by brain (1.4), gastrointestinal tract (1.3), kidney (0.3), and heart (0.1). In the day-old chick, liver and gastrointestinal tract have high thiamine contents (3.2 and 2.9), followed by brain (1.1), yolk (0.5) and heart (0.3). These values are compared with those previously reported for embryo and adult tissues.

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